TICK FEEDING AND THE DEVELOPMENT OF IMMUNITY TO *HYALOMMA ANATOLICUM ANATOLICUM*

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DECLARATION

I, Harsharnjit S. Gill, declare that this thesis was composed by me and that the work described therein was my own.

DEDICATION

To Gurjit and Navi for their love
and enduring patience, and my parents
for their inestimable contribution
to my education.

ABBREVIATIONS USED IN THE TEXT

ATP-ase	adenosine triphosphatase
Blot	Nitrocellulose paper having proteins electrophoretically blotted onto it.
CBH	Cutaneous basophil hypersensitivity
CoF	Cobra venom factor
CON-A	Concanavalin-A
DH	delayed hypersensitivity
IEF	isoelectric focusing
GARP	Peroxidase conjugated goat anti-rabbit IgG
MBP	major basic protein
PAS	Periodic acid-Schiffs reaction
PBS	phosphate buffered saline
PBST	phosphate buffered saline + Tween
PVP	polyvinylpyrrolidone
SGA	salivary gland antigen
SGE	salivary gland extract
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis

ABSTRACT

A study on the role of the salivary glands of Hyalomma anatolicum anatolicum in feeding, acquisition of host resistance and the cutaneous reactions to tick feeding has been made.

To investigate the sequential structural changes occurring in the salivary glands during feeding and the nature of potential secretory products, light and electron microscopy studies were coupled with histochemical tests on salivary glands and tick feeding sites. The salivary glands of H. a. anatolicum consisted of three types of acinus (I, II and III) in females and an additional type IV acinus in males. The ultrastructural features of the peripheral cells of the type I acinus supported the hypothesis that these acini secrete concentrated salts during questing stages of the life cycle to absorb water from unsaturated air. There were five granular cell types (a, b, c_1-c_3) in the type II acinus, three (d, e, f) in the type III acinus and one (g) in the type IV acinus.

The attachment cement of H. a. anatolicum was lipoprotein in nature and appeared to have been derived from the a cells of type II, and d and e cells of type III acini, the secretory granules of which had similar histochemical properties. A strong aminopeptidase and moderate acid phosphatase activity was also found localized in the attachment cement. In addition, deposits of glycoprotein and non-specific esterase materials, probably derived from the b and/or c cells of type II acini were located in the feeding lesion.

The interstitial cells which were insignificant in unfed ticks became more distinct during feeding. In females the interstitial

cells of type III acini enlarged markedly to form a basolateral labyrinth with the transformed f cells forming a water excretory unit during the rapid phase of engorgement.

Saliva and salivary gland extracts from 96 hour fed female ticks were fractionated by SDS-PAGE, immunoblotted and developed by autoradiography and enzyme labelled antibody to identify salivary protein involved in the induction of resistance to tick feedings. Sera from hypersensitized rabbits recognised nine antigenic proteins in the saliva and 17 in the salivary gland extracts. Antigenic proteins ranged in molecular weight from 14,400 to 130,000 daltons. All the antigens thus identified were glycoproteins. In addition, antigen I (molecular weight 130,000 daltons) showed acid phosphatase, and antigen III (molecular weight 96,000 daltons) both non-specific esterase and aminopeptidase activity. Intradermal inoculation of antigens I, II, III, saliva and salivary gland extracts into tick-exposed rabbits elicited immediate and delayed hypersensitivity reactions.

Detailed sequential quantitative histological analysis of tick feeding sites following primary and tertiary feedings was made to gain an insight into cellular interactions inimical to ticks. On primary infestation the cellular infiltrate at H. a. anatolicum feeding sites in rabbits and cattle was dominated by neutrophils followed by mononuclear cells. The infiltration of eosinophils and basophils at tick feeding sites was an early event in rabbits (24 hours) as compared to cattle (144 hours).

On tertiary infestation the tick feeding sites in both rabbits and cattle were characterized by massive degranulation of mast-cells

and basophils. Comparing the nature and magnitude of cellular infiltrate at tick feeding sites in rabbits and cattle, basophils appeared to be the major effectors of resistance in this system. The stronger expression of resistance in cattle corresponded with a high level of cutaneous basophil response (6-23%) as compared to rabbits (3-9%). The possible mechanisms by which the mediators released by degranulating cells mediate resistance are suggested and discussed.

CHAPTER ONE

INTRODUCTION

Ticks are probably the most important ectoparasites affecting the livestock industry in the tropical, subtropical and temperate regions of the world. On a global basis the losses associated with direct parasitization by ticks and with tick-borne diseases are staggering (Steelman, 1976).

The ixodid ticks have three stages in their life cycle: the larval stage, the nymphal stage and the adult stage. To complete engorgement each instar feeds on a host for several days. It is while feeding that the ticks adversely affect their hosts in several ways: removal of blood; physical damage to skin; secondary infection of bite wounds; in some cases by toxicosis and paralysis. Superimposed upon these is their ability to act as reservoirs and vectors of a variety of animal and human diseases (protozoal, bacterial, rickettsial and viral) (Balashov, 1972) which they transmit during feeding.

Ticks of the genus Hyalomma complex are widely distributed in northern tropical, subtropical, mediterranean and temperate countries (Hoogstraal, 1956). Immature stages of Hyalomma often feed on birds, rodents and hares that are important reservoirs of pathogens, especially viruses and rickettsia. Ticks of the Hyalomma complex are known to transmit: theileriosis, equine piroplasms, CHF-Congo virus, Q fever, Japanese (mosquito-borne) encephalitis virus, Russian spring-summer encephalitis virus, Uzberkistan hemorrhagic fever virus, Pasteurella pestis and Siberian tick typhus (Hoogstraal, 1956). Hyalomma anatolicum anatolicum Koch, is economically important as a vector of tropical theileriosis (T. annulata infection) - a protozoan

disease which poses a serious threat to the introduction of exotic cattle for crossbreeding purposes in North Africa, the Middle East, Pakistan, India and southern U.S.S.R. (Robinson, 1982).

To counteract the adverse effects of ticks and tick-borne diseases, tick control measures have largely depended upon the use of chemical acaricides. However, in recent years the development of resistance to acaricides in many parts of the world, and increasing concern about the presence of chemical residues in the animal products and the effect of these chemicals on the environment have stimulated the quest for methods of tick control other than by the use of acaricides. Amongst the alternative approaches, the use of predators, the sterile male technique, parasites or pathogens have been examined without much success. In view of the situation the innate (genetic) and acquired host resistance to tick feeding need careful consideration.

Laboratory animals (Trager, 1939a; Allen, 1973; Bagnall and Rothwell, 1974) and bovines (Johnston and Bancroft, 1918; Gregson, 1941; Roberts, 1968; Hewetson, 1971) acquire resistance to tick feeding. While feeding the ixodid ticks secrete into their hosts a variety of salivary proteins, many of which stimulate the host immune system and confer protection against subsequent tick infestation. In natural conditions the ticks usually feed on previously exposed hosts and are faced with an immune inflammatory reaction at the bite site which renders that site less suitable for feeding, and also less suitable for the transmission of pathogens (Callow and Stewart, 1978; Wikel, 1980).

The cutaneous reactions at tick feeding sites in tick resistant guinea pigs are dominated by basophils (Allen, 1973), the major effectors of resistance in this system (Brown et al., 1982a). However, there have been differing accounts of the participation of basophils at tick feeding sites in tick resistant rabbits (Brossard and Fivaz, 1982; Krinsky et al., 1982) and cattle (Schleger et al., 1976; Allen et al., 1977).

A variety of immunological responses have been implicated in the acquisition of resistance to ticks - antibody (Brown et al., 1982b), complement (Wikel and Allen, 1977) and cell mediated cutaneous basophil hypersensitivity (Bagnall, 1978; Brown, 1982) and immediate hypersensitivity (Boese, 1974; Willadsen et al., 1978).

Despite an increasing literature on the immune mechanisms, the nature of antigens involved in the induction of resistance has received very little attention. There is virtually no information on the numbers and precise location of these antigens within the tick. In addition nothing is known about the nature of immunologic responses these antigens may induce, though such responses could be of extreme importance.

Attempts to immunize potential hosts artificially against ticks using crude antigens have met with limited success. Antigens used in these studies included whole tick extracts (McGowan et al., 1981) reproductive organs and gut tissues (Allen and Humphreys, 1979) and salivary glands (Brossard, 1976; Wikel, 1981). The level of protection conferred by these antigens was considerably lower than that conferred by natural tick feeding. This was probably due to the use

of poorly defined antigens. Therefore, in any attempt to develop methods for tick control based on acquired resistance (anti-tick vaccines; skin test for selection of resistant cattle), a profound knowledge of the feeding mechanisms, the nature of tick derived antigens and the pathologic factors (at tick feeding sites) inimical to ticks is essential.

The present study was carried out with the following objectives.

(i) To examine the sequence of morphological and ultrastructural changes occurring in the salivary glands of both male and female H. a. anatolicum during feeding.

(ii) To identify the potential secretory products in the salivary glands and to see if they are secreted into the host during feeding.

(iii) To isolate and characterize the antigens originating from H. a. anatolicum salivary glands, and to determine the nature of the immune response elicited by these antigens.

(iv) To define and compare the sequence of histological changes at adult H. a. anatolicum feeding sites in naive and tick resistant rabbits and cattle.

(v) To gain an insight into the cellular interactions inimical to ticks at H. a. anatolicum feeding sites in tick resistant rabbits and cattle.

(vi) To monitor the effect of acquired resistance in rabbits and cattle on tick feeding and oviposition.

The anticipated knowledge gained from this study would also help to understand: how an arthropod obtains its blood meal and the nature of the ingested food; by what means the antigenic/pathogenic material, once inoculated, is processed by the host cells and spreads into the tissues.

CHAPTER TWO

REVIEW OF LITERATURE

CONTENTS

2.1 TICK SALIVARY GLANDS

Tick salivary glands have been the focus of intensive investigations in recent years. The application of semi-thin sectioning coupled with histochemistry (Binnington, 1978; Binnington and Stone, 1981; Walker et al., 1984) and electron microscopy (Coons and Roshdy, 1973; Meredith and Kaufman, 1973; Megaw and Beadle, 1979; Fawcett et al., 1981a,b; Krolak et al., 1982; Walker et al., 1984) has revealed that the structure of ixodid salivary glands is more complicated than was originally appreciated.

The complexity of the salivary glands is not surprising since they are known to perform such varied functions as secretion of attachment cement (Moorhouse, 1969), secretion of anticoagulants (Ross, 1926; Foggie, 1959), pharmacological agents (Dickinson et al., 1976; Higgs et al., 1976; Shemesh et al., 1979) and elimination of excess fluid and ions during feeding (Gregson, 1967; Tatchell, 1967; Meredith and Kaufman, 1973).

The salivary glands consist of a pair of glandular masses, situated laterally in the body cavity and extending caudally to the level of the spiracles. The main salivary duct opens anteriorly into the salivarium and extends posteriorly giving rise to secondary and tertiary branches. Acini open by means of short efferent ducts throughout the duct system. The ixodid salivary glands consist of two morphologically distinct acinar types: agranular and granular acini. These acini are further classified as the agranular type I, and granule secreting acini types II and III in females with an additional type IV in males (Till, 1961; Balashov, 1972; Binnington,

1978; Walker et al., 1984) except sub-family ixodinae (Balashov, 1972; Binnington and Stone, 1981).

The type I acini are confined to the anterior region of the gland and open directly into the main duct through short efferent ducts, lacking a valve-like structure. These acini were thought to be involved in osmoregulation, due to their ultrastructural similarities to fluid transporting epithelia (Kirkland, 1971; Balashov, 1972). However, involvement with an activity other than osmoregulation has been argued on the following basis: their presence in small numbers, and their distribution in the anterior part of the gland (Meredith and Kaufman, 1973; Megaw and Beadle, 1979); the presence of type I acini in males which do not excrete excess fluids (Coons and Roshdy, 1973) and in argasids which eliminate excess fluids through coxal glands (Lees, 1946). Recent studies have postulated that they are involved in the secretion of concentrated salts in order to absorb water vapour from unsaturated air (McMullen et al., 1976; Rudolph and Knülle, 1978). In addition there are good reasons for believing that the adlumenal interstitial cells (water cells of Meredith and Kaufman, 1973) of acini II and III which develop greatly during feeding are responsible for the excretion of excess fluids during feeding (Meredith and Kaufman, 1973; Megaw and Beadle, 1979).

Krolak et al. (1982) studied in great detail the ultrastructure of the type I acini of Amblyomma americanum. They described four different types of cells: pyramidal, central, peritubular and constrictor cells. The pyramidal cells were characterized by extensive

basal infoldings with closely associated mitochondria and were similar to those described in Dermacentor variabilis (Coons and Roshdy, 1973), Boophilus microplus (Megaw and Beadle, 1979) and Rhipicephalus appendiculatus (Walker et al., 1984). The central cell had electron lucent cytoplasm with few mitochondria, and made contact with the duct lumen through an opening in the constrictor cell.

The granule-secreting acini types II and III, each consist of at least three different granular cell types which are similar in both male and female ixodid ticks. These granular cell types are separated from each other by interstitial cells. Type II acini consist of three different types of granular cells a, b and c. These cells are arranged in a concentric manner around a central lumen which communicates with the lobular duct through a cuticular valve. There are one or two a cells present on either side of the cuticular valve. In the unfed ticks these cells are packed with complex secretory granules. The b cells are found between the a and c cell types, the latter occupying the fundus of the acinus. The c cells have been further classified into types c_1 to c_3 (Coons and Roshdy, 1973) on the basis of their ultrastructure and c_1 to c_4 (Binnington, 1978) on the basis of their histochemical properties. The type III acini contain three different cell types: d, e and f. Like a cells of acinus II, one or two d cells lie adjacent to the valvular duct. Next to the d cells are four to five large sized e cells. Lying in the fundus region of acinus III are a group of small cells, the f cells, with few or no granules in the unfed tick.

Acinus IV is found only in males and contains only one type of granular cell, the g cell (Binnington, 1978).

However, there is still considerable disagreement on the numbers and types of granular cells. Except for a cells of type II acini, and d and e cells of type III acini, which are identified by their complex secretory granules, the description of various authors of the b/c cells do not match up.

Recent studies have shown that there are seven different granular cell types in male D. variabilis (Coons and Roshdy, 1973), nine types in female B. microplus, ten types in male B. microplus (Binnington, 1978), eight types in female R. appendiculatus, nine types in male R. appendiculatus (Walker et al., 1984) and four types in Ixodes holocyclus (Binnington and Stone, 1981).

The secretory granules of cell types a of acinus II, and d and e of acinus III contain lipoproteins in B. microplus (Binnington, 1978) and R. appendiculatus (Walker et al., 1984). Cells with similar histochemical properties have also been described in Haemaphysalis spinigera (Chinery, 1965) and Hyalomma asiaticum (Balashov, 1972) and were suggested to be the precursors of attachment cement. The cement cone of B. microplus consisted of an internum of lipoproteins and a cortex of a carbohydrate-protein complex (Moorhouse and Tatchell, 1966). Cells a, d and e in B. microplus (Binnington, 1978) and R. appendiculatus also exhibit a strong phenol oxidase activity, indicating a possible tanning process of the cement cone analogous to insect cuticle (Binnington, 1978). In contrast to B. microplus and R. appendiculatus, the cell types a, d and e of male D. variabilis (Coons and Roshdy, 1973) and I. holocyclus (Binnington and Stone, 1981) stained to varying intensities for polysaccharides.

The remaining cell types - *b*, c_1 to c_3 of acinus II, *f* cells of acinus III and *g* cells of acinus IV - stain for glycoproteins and various enzymes in *B. microplus* (Binnington, 1978) and *R. appendiculatus* (Martins, 1977; Walker *et al.*, 1984). Non-specific esterase activity has been demonstrated in the acinar types I and II and the apical membranes of type III acini, in both *B. microplus* and *R. appendiculatus* by these workers. Geczy *et al.* (1971) suggested that these esterases might increase vascular permeability by promoting mast-cell degranulation. An esterase purified from larval homogenates of *B. microplus* (Willadsen *et al.*, 1978) did not induce any visible oedema when injected into non-immune hosts. However, its demonstration at *B. microplus* larval feeding sites and its rapid removal in immune hosts (Tracey-Patte, 1979) are suggestive of an important role of these enzymes for successful feeding. In addition, Schleger and Lincoln (1976) demonstrated the presence of aminopeptidase in the attachment cone and adjacent epidermis. Aminopeptidases have also been demonstrated in the salivary glands of *R. appendiculatus* (Martins, 1977; Walker *et al.*, 1984).

The cells *b* and *c*, which synthesize and secrete their products throughout feeding, have been suggested as the source of anticoagulants in *H. spinigera* (Chinery, 1965). An anticoagulant activity has been demonstrated in the salivary glands of *I. holocyclus* (Ross, 1926; Kaire, 1967), *D. andersoni* (Hoeppli and Feng, 1931) and *Ixodes ricinus* (Foggie, 1959). The saliva of *B. microplus* lacks such an activity (Tatchell, 1969). However, Willadsen and Riding (1979) purified a proteolytic enzyme inhibitor with allergic activity which also had an anticoagulant property.

In addition, tick salivary glands have been found to be the source of prostaglandins in B. microplus (Dickinson et al., 1976; Higgs et al., 1976) and Hyalomma anatolicum excavatum (Shemesh et al., 1979), histamine-like and histamine antagonists in Rhipicephalus sanguineus sanguineus (Chinery and Ayitey-Smith, 1977) and cytolysins in I. ricinus (Foggie, 1959; Arthur, 1965). These agents appear to provide a less destructive mechanism of obtaining a blood meal over a period of several days. However, from some of these findings it is very difficult to draw a valid conclusion, as the proteolytic enzyme inhibitor isolated from B. microplus also blocks the action of complement (Willadsen, 1980) and could be a possible means of evading the host immune system. In addition prostaglandins have been found to be involved with reproduction in some insects (Destephano and Brady, 1977).

2.1.1 Changes During Feeding

Attachment of the tick to the host appears to stimulate a series of structural changes, especially in the granular acini of the salivary glands, which are reflected by the changing function of the glands during feeding. The type I acini do not change in size or structure during feeding, although the glycogen-like material (Megaw and Beadle, 1979; Walker et al., 1984) and lipid droplets (Walker et al., 1984) present in unfed ticks are not seen during later stages of feeding.

Acinar types II and III undergo significant changes during feeding. In B. microplus (Binnington, 1978; Megaw and Beadle, 1979) and R. appendiculatus (Walker et al., 1984) the a cells of acinus II,

and d and e cells of acinus III shrivel rapidly during early feeding and are almost devoid of granular activity by 72 hours after attachment. It is important to note that the secretion of these granules corresponds with the deposition of attachment cement (Moorhouse, 1969). The b and c cells show a progressive increase in size and appear to secrete their products throughout feeding in both B. microplus and R. appendiculatus. The esterase activity present in all these cells in unfed ticks is no longer seen after 72 hours of feeding in B. microplus.

The f cells of acinus III which do not have any granules in the unfed ticks become active after 12 to 24 hours of feeding and have lost their secretory ability by 72 hours (Binnington, 1978). The interstitial cells which are insignificant in unfed ticks enlarge and fill the acini during feeding (Meredith and Kaufman, 1973). Ultrastructural studies have revealed that these interstitial cells are present in two tiers. Due to their similar ultrastructure and respective positions in the acini, Fawcett et al. (1981b) called these the adlumenal interstitial cell (one bordering the lumen), and ablumenal interstitial cells (others having no direct access to the lumen), analogous to the cap and water cells of Meredith and Kaufman (1973). There is only one adlumenal interstitial cell in A. americanum which winds its way around the acinar lumen (Krolak et al., 1982). They suggested that this cell helps in propelling secretions from the acinar lumen to the duct.

The type III acini, responsible for the excretion of excess fluids and ions, undergo dramatic changes during feeding (Meredith

and Kaufman, 1973). As the tick approaches engorgement the adlumenal interstitial cells along with transformed f cells form an extensive labyrinthine system. Meredith and Kaufman (1973) and Megaw and Beadle (1979) attributed the function of fluid transport to the ablumenal interstitial cells whereas Fawcett et al. (1981b) suggested that the f cells also play an important role in fluid transport, as these cells present a greatly amplified surface area to the extracellular labyrinth.

In contrast to female ticks, type II and III acini in males do not change during feeding. There is a progressive increase in the granular activity of g cells of acinus IV, which becomes almost the largest acini in feeding males. The secretions of these acini were suggested to be involved in reproduction (Feldman-Muhsam et al., 1970).

2.2 ATTACHMENT TO THE HOST

Having found a suitable site for feeding the initial penetration is achieved by sweeping and cutting motions of the chelicerae. This is followed by the secretion of attachment cement. In the genera Amblyomma, Hyalomma and Aponomma, the long mouthparts penetrate deeply into the dermis and are ensheathed along their entire length by the attachment cement (Moorhouse, 1969). In Rhipicephalus, Boophilus and Haemaphysalis, with comparatively short mouthparts, the penetration and secretion of the cement is superficial. In some members of the genus Ixodes the mouthparts penetrate deeply into the dermis and are held in situ by the deformed host tissues. Stevens

(1968) found an aggregation of collagen around the mouthparts of I. ricinus and suggested an active synthesis of collagen, although the fibroblasts which are known to secrete collagen were absent. She proposed that it is possible that the tick provides the stimulus for polymerization of the collagen precursors in the skin. In contrast to Stevens, Pavlovsky and Alfeeva (1941, 1949; cited by Stevens, 1968) reported the reorientation of collagen fibres at the point of entry of I. ricinus and accumulation of collagen at the point of penetration of Hyalomma respectively. Collagen bundles were seen embedded in the attachment cement of Haemaphysalis flava, Ixodes persulcatus and Ixodes japonensis (Saito and Ohara, 1961). On the other hand, Tatchell and Moorhouse (1968) suggested that the collagen was digested in the formation of the lesion following B. microplus attachment.

The main function of the cement is to provide secure attachment, to help the tick to create a negative pressure for the suction of tissue fluids (Saito et al., 1960) and to protect adjacent tissues from the lytic effect of salivary enzymes (Arthur, 1970). Tatchell and Moorhouse (1968) suggested that the cement of B. microplus was inactive. On the other hand, Gregson (1970) demonstrated that lymphocytes from sensitized individuals responded markedly to tick saliva and cement. Theis and Budwiser (1974) observed a dermal reaction consisting of neutrophils and mononuclear cells when they left the cement and mouthparts of R. sanguineus in situ. However, from the histochemical nature of cement it is reasonable to speculate a foreign body type reaction by the host.

2.3 DEVELOPMENT OF THE FEEDING LESION

There have been differing accounts of the role of salivary secretions in the formation of the feeding lesion. Tatchell (1969) hypothesized that the ixodid genera with deeply inserted mouthparts supported by attachment cement are likely to secrete powerful proteolytic enzymes as any host tissue destruction will take place at the tip of the hypostome, whereas superficially attaching ticks are unlikely to secrete enzymes that could endanger their attachment. Accordingly such ticks must inflict damage by more subtle means.

Strong lytic enzymes have been suggested to occur in the saliva of I. holocyclus (Moorhouse, 1973). Tissue lysis following Hyalomma rufipes feeding has been attributed to lytic salivary enzymes (Arthur, 1973). However, in B. microplus specific vascular damage is caused by the tick saliva while the tissue damage results from the host response (Tatchell and Moorhouse, 1970). A small focus of tissue destruction caused by mechanical damage at the tip of the hypostome, enlarges rapidly due to infiltration of granulocytes and the necrosis of adjacent tissues. Neutrophil dependent tissue destruction was demonstrated by Tatchell and Moorhouse (1970) who induced neutropenia with nitrogen mustard. In neutrophil-depleted animals the feeding lesions were insignificant and lacked tissue destruction; nevertheless ticks feeding on such hosts laid viable eggs. Further support to Tatchell and Moorhouse's hypothesis comes from Berenberg et al. (1972) who demonstrated that injury caused by ticks was non-immunologic and was neutrophil-dependent. He demonstrated the chemotaxis of neutrophils to tick feeding sites following activation of complement by saliva of D. variabilis.

2.4 HISTOLOGY OF TICK FEEDING SITES ON NAIVE HOSTS

The nature of the cellular infiltrate at tick feeding sites varies greatly depending upon the tick and host species, and time after attachment.

Tatchell and Moorhouse (1968) studied the histology of B. microplus feeding sites in zebu (Bos indicus) and European breeds (Bos taurus) of cattle and found that the definitive lesions were similar in all the hosts. They consisted predominantly of neutrophils, some lymphocytes, and erythrocytes in an area of heavily infiltrated collagen. Intensity of neutrophil infiltration was accompanied by the destruction of collagen and development of a feeding cavity. There was increased incidence of blockage of capillaries as the feeding advanced.

Following the attachment of R. sanguineus on dogs (Theis and Budwiser, 1974) and A. americanum on guinea-pigs (Brown and Knap, 1980), neutrophils were the predominant cells followed by mononuclear cells. However, at I. holocyclus feeding sites on guinea-pigs neutrophil levels were high initially (34%), but quickly subsided (6-15%) as the feeding advanced. On the other hand, mononuclear cells dominated the cellular infiltrate at Ixodes uriae (Eveleigh et al., 1974), Ixodes dammini (Krinsky et al., 1982) and I. holocyclus (Brown et al., 1984) feeding sites in chickens, rabbits and guinea-pigs respectively.

Stevens (1968) found accumulation and breakdown of mast-cells at I. ricinus feeding sites, and associated it with oedema. Mast-cell degranulation was also observed at R. sanguineus tick feeding

sites on unsensitized dogs (Theis and Budwiser, 1974). This non-immunologic degranulation of mast-cells was attributed to mechanical disruption during initial penetration and to tick saliva.

Degranulation of mast-cells results in the release of chemo-attractants for other inflammatory cells and pharmacological media-tors capable of increasing vascular permeability, thus making avail-able large quantities of blood during the final rapid engorgement phase (Tatchell and Moorhouse, 1968).

A marked infiltration of eosinophils was observed in bovine skin parasitized by B. microplus (Tatchell and Moorhouse, 1968) and I. holocyclus (Allen et al., 1977). It was suggested that the oral secretions of these ticks might have a built-in chemotactic activity for eosinophils. At R. appendiculatus feeding sites on guinea-pigs eosinophils were the predominant cell in early (6 hours) and late (72 hours) feeding lesions (Brown et al., 1983). In contrast, eosinophils were almost absent at R. sanguineus feeding sites on dogs (Theis and Budwiser, 1974) and I. holocyclus feeding sites on guinea-pigs (Brown et al., 1984).

In guinea-pigs infested with Dermacentor andersoni (Allen, 1973) and R. appendiculatus (Brown et al., 1983), a large number of basophils infiltrated the skin towards the end of feeding. At R. appendiculatus feeding sites basophils were first observed at 72 hours after attachment and constituted 78% of the cellular infil-trate at 96 hours. On the other hand, the basophil infiltrate at I. ricinus (Brossard and Fivaz, 1982) and I. dammini (Krinsky et al., 1982) feeding sites on rabbits was comparatively smaller, and negli-gible at B. microplus (Schleger et al., 1976) feeding sites on cattle.

2.5 HISTOLOGY OF TICK FEEDING SITES ON TICK RESISTANT HOSTS

2.5.1 Bovine Hosts

Acquisition of resistance to tick feeding by bovines has been suggested by several workers (Johnston and Bancroft, 1918; Gregson, 1941; Roberts, 1968; Hewetson, 1971; Wagland, 1975; Allen et al., 1977). It is well established that zebu (Bos indicus) are less susceptible to tick infestation than European cattle (Bos taurus). The higher level of resistance shown by zebu cattle is thought to be due to "adaptation tolerance" resulting from their co-evolution with the ticks (Tatchell, 1969). However, there are differences in susceptibilities of individuals within the breed (Wilkinson, 1955).

Experimental studies have shown that both zebu and European breeds of cattle acquire resistance to tick infestation and that the ability to acquire a particular degree of resistance is heritable (Wharton et al., 1970).

In an attempt to obtain an idea of the nature of the host response, the histology of tick feeding sites on resistant bovines has been studied by Tatchell and Moorhouse (1968), Schleger et al. (1976) and Allen et al. (1977). Larval feeding sites on resistant hosts were characterized by capillary dilation, oedema and infiltration of eosinophils. The magnitude of eosinophil infiltration, degranulation and mast-cell disruption were all significantly greater at larval feeding sites on hosts of high resistance than those of low resistance (Schleger et al., 1976). They further suggested that the massive degranulation could be due to reaction of specific antigen with homocytotropic antibody in addition to mechanical disruption,

action of saliva or complement activation (Movat, 1971). Mast-cell degranulation is an important event in the initiation of an immediate hypersensitivity reaction (Keller and Schauwecker, 1972) and has been hypothesized to have a protective function against arthropod bites (Stebbings, 1974).

Basophils infiltrated in large numbers into the skin of cattle following tertiary infestation by I. holocyclus (Allen et al., 1977). By four hours after attachment most of the basophils were degranulated and had led to infiltration by a new wave of eosinophils. However, only a few basophils infiltrated at B. microplus feeding sites in cattle (Schleger et al., 1976). In the absence of a strong cutaneous basophil response the translocation of histamine by eosinophils to tick attachment sites (Schleger et al., 1981) could be an important event. The amount of histamine available locally correlated directly with the degree of resistance and the intensity of hypersensitivity response (Willadsen et al., 1979). The hypersensitivity reaction was partially modified following treatment with antihistamine drugs (Tatchell and Bennett, 1969; Wikel, 1982), which suggested that histamine was an important mediator of resistance. Histamine release at the tick attachment site stimulates grooming (Schleger et al., 1976) which has been found to result in the rejection of up to 50% of larvae initially stabilized on the host (Koudstaal et al., 1978). In vivo and in vitro experiments have shown that histamine has a direct effect on tick attachment behaviour (Kemp and Bourne, 1980). How this and other mediators affect resistance is, however, not clear.

2.5.2 Laboratory Hosts

Acquisition of resistance to tick feeding by laboratory animals was first reported by Trager (1939a). He observed an intense inflammatory response at D. variabilis feeding sites in tick-resistant guinea-pigs. He hypothesized that the manifestation of resistance was by walling-off the feeding lesion, thus preventing access to dermal blood vessels. However, recent studies have shown that the mechanism of resistance is much more complex. In the ixodid tick-guinea-pig system, basophils have been shown to be the major components of the cellular infiltrate in immune cutaneous responses to tick feedings (Allen, 1973; Bagnall, 1978; Brown and Knap, 1981). These cells are not residents of skin and are recruited from the bone marrow via the blood to participate in local immunologic reactions.

Basophils accumulated in large numbers beneath the mouthparts and in epidermal vesicles in resistant guinea-pigs challenged with larval D. andersoni (Allen, 1973). An ability to produce epidermal vesicles was related to the resistant status of the host (Trager, 1939a). In addition Allen (1973) also noticed a significant infiltration of eosinophils at the tick feeding sites. However, due to basophils being the dominant cells Allen (1973) called it a cutaneous basophil hypersensitivity reaction analogous to the one described by Dvorak et al. (1970).

Basophils and eosinophils were the principal host cells involved in the acquisition of resistance by guinea-pigs to A. americanum (Brown and Knap, 1981; Brown and Askenase, 1981) and the

magnitude of basophil infiltration was directly related to the level of tick rejection. Following R. appendiculatus feedings on sensitized guinea-pigs, basophils (60-91%) dominated the cellular infiltrate at all observation times, followed by eosinophils (7-21%) (Brown et al., 1983). On the other hand, eosinophils were almost absent from I. holocyclus feeding sites on actively and passively sensitized guinea-pigs (Brown et al., 1984), while basophils (72-79%) were the predominant cells from 48 to 72 hours after tick attachment and coincided with the timing of significant tick rejection. This suggests that the basophils alone mediate resistance in this tick-host system.

Challenge feeding sites in guinea-pigs resistant to R. sanguineus were characterized by predominant infiltration of mononuclear cells (58%) with moderate proportions of basophils (24%) (Brown and Askenase, 1981). In contrast, mononuclear cells and eosinophils were the predominant cells at I. dammini feeding sites on hypersensitized rabbits that failed to express resistance (Krinsky et al., 1982). However, basophils were the major cell types at I. dammini feeding sites in resistant guinea-pigs (Krinsky et al., 1982). This further suggests that basophils are essential for the expression of resistance.

All these workers also reported a massive degranulation of mast-cells and basophils at tick feeding sites on guinea-pigs following challenge infestation. Brown et al. (1982a) suggested that the degranulation might have been due to the interaction of tick salivary antigens with homocytotropic antibody bound to basophils. Progressive sensitization of basophils to salivary antigens has been demonstrated by Brossard et al. (1982). Degranulation leads to the release of

histamine and other vasoactive amines and chemotactic factors for eosinophils (Askenase, 1977). The histamine concentration in the tissues at tick challenge sites was significantly greater in resistant animals than in naive animals (Wikel, 1982). In an *in vitro* system the normal feeding behaviour of *D. andersoni* was disrupted on the addition of histamine and 5-hydroxytryptamine to the feeding medium (Paine *et al.*, 1983).

From these studies it is logical to conclude that if the vasoactive amines are protective substances then the mediators produced by eosinophils might decrease the host immunity to ectoparasites. However, a protective function of eosinophils has been demonstrated in a number of host-parasite systems (Grove *et al.*, 1977; McLaren *et al.*, 1978; Butterworth *et al.*, 1979; Kierszenbaum *et al.*, 1981). It is possible that the eosinophils have both these properties - firstly, to dampen the inflammation (Weller and Goetzl, 1979) and secondly to damage the gut epithelia of the tick as suggested by Brossard and Fivaz (1982).

A definitive and important role of basophils and eosinophils in the acquisition of resistance to ticks was demonstrated by Brown *et al.* (1982a). Specific depletion of basophils by anti-basophil serum resulted in the blockage of resistance to *A. americanum*. Similarly, the administration of anti-eosinophil serum resulted in the partial expression of resistance. In addition eosinophil levels in basophil-depleted animals were reduced at tick feeding sites, suggesting basophil-dependent recruitment of eosinophils. This study indicated that the basophils and to a lesser extent eosinophils were responsible for the expression of resistance in the guinea-pig *A. americanum* system.

2.6 IMMUNOLOGIC NATURE OF RESISTANCE

Involvement of immunologic components in the acquisition of resistance has been suggested by several authors for different tick-host systems (Trager, 1939a; Allen, 1973; Boese, 1974; Bagnall and Rothwell, 1974; Wikel and Allen, 1976a,b; Brown et al., 1982b). Trager (1939a) furnished the first evidence that the acquisition of resistance to D. variabilis had an immunologic basis. Later Allen (1973) substantiated Trager's observations when he showed that the resistance to D. andersoni could be blocked by administration of methotrexate - an immunosuppressant.

The host response to the ixodid tick is manifested by various types of immunological responses: immediate hypersensitivity, antibody and complement mediated, and delayed/cutaneous basophil hypersensitivity reactions.

2.6.1 Immediate Hypersensitivity

The immediate hypersensitivity to an arthropod bite has a protective function (Stebbings, 1974). There is evidence that the immediate hypersensitivity reactions are at least partially responsible for the expression of resistance to larval B. microplus. Resistance was first equated with hypersensitivity by Riek (1962), when he observed increased histamine levels in the blood of resistant bovines. Intradermal inoculation of eggs and larval extracts gave immediate oedematous reactions and the ability to produce these reactions was passively transferred with immune serum (Riek, 1958). Allergens purified from unfed B. microplus larvae gave an oedematous reaction. The ability to produce these reactions correlated with the

degree of resistance to ticks (Willadsen and Williams, 1976; Willadsen et al., 1978; Willadsen and Riding, 1979).

Larval B. microplus feeding sites on resistant hosts were characterized by massive degranulation of mast-cells and infiltration of eosinophils (Schleger et al., 1976). Degranulation of mast-cells led to the release of histamine and other pharmacologically active substances (Askenase, 1977). Histamine has been shown to have a direct effect on tick attachment (Kemp and Bourne, 1980) and may induce reflex grooming, the major effector of larval losses in resistant hosts (Koudstaal et al., 1978).

Immediate hypersensitivity has been shown to be involved in many other tick-host systems (Allen, 1973; Allen et al., 1977; McGowan et al., 1979). Ixodes holocyclus feeding sites in sensitized cattle showed increased eosinophil and basophil infiltration and degranulation (Allen et al., 1977) which are typical of allergic reactions (Weller and Goetzl, 1979).

Homocytotropic antibodies against I. ricinus (Brossard and Girardin, 1979) and Haemaphysalis leporispalustris (Boese, 1974) were demonstrated in rabbits during the course of multiple infestations. McGowan et al. (1979) further demonstrated that the development of resistance by cotton tails and rabbits to H. leporispalustris correlated with the development of skin-sensitizing antibodies.

These studies indicate that immediate hypersensitivity is at least partially responsible for the expression of resistance in these tick-host systems. However, the number of tick-host systems investigated is very small. More studies should be conducted to elucidate the role of this mechanism in the expression of resistance to ixodid ticks in general.

2.6.2 Antibody and Complement Mediated Reactions

The involvement of humoral factors in the expression of resistance to ixodid ticks has been reported several times. Trager (1939a) demonstrated the acquisition of resistance to D. variabilis larvae by guinea-pigs after a single infestation. Partial resistance was passively transferred with immune serum to susceptible guinea-pigs. However, the expression of resistance was inconsistent, thus making it difficult to draw any valid conclusions. Trager (1939b) also demonstrated the presence of complement fixing antibodies to larval extracts of D. andersoni.

The acquisition of resistance by rabbits to I. ricinus is also partially antibody mediated, as transfer of serum from resistant to susceptible hosts resulted in reduced engorgement weights in feeding female ticks as compared to unexposed controls (Brossard, 1977). However, there was no effect on the time to engorgement. Similarly, plasma from cattle highly resistant to the tick B. microplus conferred significant protection against tick challenge, whereas plasma from hosts of low resistance had no significant effect compared with plasma from unexposed donors (Roberts and Kerr, 1976).

Passive transfer of immune serum conferred significant protection against A. americanum and R. sanguineus (Brown and Askenase, 1981), thus further suggesting the involvement of humoral factors. Resistance to R. appendiculatus was readily transferred with serum from donors sensitized by a single prior infestation. Doses as small as 0.5 ml conferred significant protection against challenge infestation (Askenase et al., 1982).

On the other hand, attempts to transfer resistance to I. holocyclus (Bagnall and Rothwell, 1974) in guinea-pigs using immune serum were unsuccessful. This suggested a more important role for cell mediated components in the expression of resistance in this tick-host system.

Precipitating antibodies were demonstrated in the sera of rabbits resistant to the bites of Haemaphysalis longicornis (Fujisaki, 1978). Fractionation of immune serum by sephadex G-200 chromatography showed immunoglobulins of 7S classes. Both precipitating and complement fixing antibodies were demonstrated in rabbits subjected to series of H. longicornis (Fujisaki et al., 1980), H. a. excavatum and R. sanguineus (Kohler et al., 1967; cited by Balashov, 1972) infestations. The existence of a negative correlation between the antibody titre and engorgement weights suggested a significant role of humoral components in the expression of resistance to H. longicornis. However, there was no relationship between the production of humoral antibodies and the numbers of ticks used in individual infestations or the intervals between them.

Circulating anti-I. ricinus antibodies of IgG class were demonstrated by Bowessidjaou et al. (1977) and Brossard and Girardin (1979). The results of Bowessidjaou et al. (1977) also showed that the titre of precipitating antibody response and the degree of resistance were not closely linked, as the antibody titres did not increase after a second infestation although the host became progressively more immune.

Using indirect immunofluorescence and immunoelectrophoresis Brossard (1976) demonstrated the presence of specific and non-specific antibodies against salivary glands of B. microplus in serum from resistant cattle and found a positive causal relationship between antibody titre and the development of resistance. There was an increase in the total serum gamma globulin following tick infestation. Contrary to Brossard, Willadsen et al. (1978) failed to find a positive correlation between the levels of precipitating antibody and the degree of resistance, as higher antibody titres were found in cattle of low resistance.

The involvement of complement in resistance to ticks was demonstrated by Wikel and Allen (1977). The depletion of complement in tick resistant animals by the administration of cobra venom factor (CoF) blocked the expression of resistance. The blockage of resistance was manifested by an increase in the numbers and weights of larvae engorged, and reduction in basophil numbers at tick feeding sites in resistant animals treated with CoF. Subsequently, Wikel (1979) provided further evidence that the alternative pathway of complement activation was involved in the expression of resistance to D. andersoni. Complement C_4-deficient guinea-pigs acquired and expressed resistance in a fashion similar to animals with a fully functional complement system. However, the results should be viewed with caution as complement activation has been reported to occur both in immunologic and non-immunologic ways following parasitic infections (Berenberg et al., 1972; Santoro et al., 1979).

2.6.3 Delayed Hypersensitivity

Delayed skin hypersensitivity appears to be a common response to infestation with arthropod parasites (Allen, 1966; Benjamini and Feingold, 1970). Gregson (1970) demonstrated that lymphocytes from individuals sensitized to D. andersoni responded to both tick saliva and cement, though the responsiveness was greater to the attachment cement.

Wikel et al. (1978) showed that an intradermal injection of salivary gland antigen (SGA) elicited strong antigen-specific delayed skin reactivity in tick resistant guinea-pigs. A non-specific immediate reactivity was also observed in both tick resistant and control animals, probably suggesting the presence of a vasoactive moiety in the salivary glands. Antigen-specific in vitro lymphocyte responsiveness appeared two to four days after the end of primary infestation and persisted until the end of challenge infestation, and corresponded with the infiltration of basophils at tick feeding sites. Peak lymphocyte responsiveness was observed 24 hours after the initiation of the second infestation and at a time when large numbers of basophils were attracted to the tick attachment site. Similar observations were made by Bagnall (1975; cited by Willadsen, 1980) while investigating an I. holocyclus-guinea-pig system. Larval extracts elicited an immediate reaction on intradermal inoculation in both the immune and control guinea-pigs, the reactions being more pronounced in resistant hosts. Histological examination of reaction sites revealed accumulations of lymphocytes and macrophages in all animals and significant infiltration of basophils and eosinophils in immune hosts.

Wikel and Osburn (1982) observed delayed skin reactions to SGA in adult cattle and calves which had experienced tick infestations. However, there was no relationship between the cutaneous responsiveness to SGA and the number of tick infestations. Peripheral blood lymphocytes harvested at the end of the third or fourth tick infestation exhibited antigen-specific *in vitro* lymphocyte blastogenesis. However, none of the authors investigated whether T and/or B cells were stimulated by specific salivary antigens.

In both *D. andersoni* and *I. holocyclus*-guinea-pig systems resistance was adoptively transferred with sensitized lymph node cells (Bagnall, 1978; Wikel and Allen, 1976a; Askenase *et al.*, 1982) and resistance to *D. andersoni* was more readily transferred with T-cell enriched populations (Wikel, 1976). Passive transfer of sensitized peritoneal exudate cells to susceptible guinea-pigs conferred significant resistance to *A. americanum* and *R. sanguineus* (Brown and Askenase, 1981).

Interestingly, in all these tick-host systems the immune rejection of ticks has been associated with massive infiltration of basophils to the tick attachment sites. These basophil-rich cutaneous reactions are typical of the cutaneous basophil hypersensitivity described by Dvorak *et al.* (1970).

Delayed hypersensitivity reactions in guinea-pigs have been divided into two types: classical delayed hypersensitivity (DH) and cutaneous basophil hypersensitivity (CBH) (Stashenko *et al.*, 1977). CBH reactions are different from classical DH, in which mononuclear cells are the predominant cells with few or no basophils (Dvorak *et al.*, 1974).

Cutaneous basophil hypersensitivity (CBH): CBH reactions are basophil-rich immune cutaneous reactions with a delayed time course. These are heterogenous in nature and involve cell mediated (Dvorak et al., 1971) and antibody dependent effector mechanisms (Askenase et al., 1975).

Tick feeding sites in resistant guinea-pigs are characterized by accumulations of large numbers of basophils (Allen, 1973; Bagnall, 1978; Brown and Askenase, 1981; Brown et al., 1984). Basophils formed a significant proportion of the cellular infiltrate at tick feeding sites in rabbits expressing resistance to I. ricinus (Brossard and Fivaz, 1982) and cattle expressing resistance to I. holocyclus (Allen et al., 1977).

Immune resistance to A. americanum and R. sanguineus is dependent on sensitized cells or immune serum (Brown and Askenase, 1981), and is associated with a cutaneous basophil response in donors and recipients. However, in both tick-host systems a greater level of protection was conferred by sensitized cells. Rejection of both tick species also had a weaker but significant humoral component. Fractionation of immune serum by gel filtration chromatography and ion exchange chromatography showed that IgG antibodies were responsible for the ability of immune serum to transfer cutaneous basophil-associated immune resistance against A. americanum (Brown et al., 1982b). Administration of highly specific anti-basophil serum resulted in the depletion of basophils from tick feeding sites and completely abrogated the expression of resistance (Brown et al., 1982a).

Both immune serum factors and sensitized cells were found to be necessary for the expression of complete resistance to I. holocyclus (Askenase et al., 1982), as immune serum or sensitized cells when given alone conferred only a moderate degree of resistance. However, the level of resistance was found to be directly related to the degree of tissue basophilia at the tick attachment site (Brown et al., 1984).

On the other hand, the cutaneous basophil response to R. appendiculatus was successfully transferred with immune serum and conferred almost complete resistance to a challenge infestation (Askenase et al., 1982). Serum doses as small as 0.5 ml transferred resistance.

Unlike R. appendiculatus, the transfer of immune serum failed to provide any protection against D. andersoni infestation (Wikel and Allen, 1976a). However, resistance was successfully transferred with viable lymph node cells and was more readily transferred with T-cell enriched populations. Wikel (1976) observed peak in vitro lymphocyte responsiveness to D. andersoni salivary antigens 24 hours after challenge infestation, the stage when Allen (1973) observed massive degranulation of basophils at D. andersoni feeding sites.

In addition the depletion of complement following CoF treatment resulted in a significant reduction in the numbers of basophils at tick attachment sites in resistant guinea-pigs (Wikel and Allen, 1977). This suggests that complement may also play an important role in chemotaxis of basophils.

All these studies suggest that the recruitment of basophils to immune cutaneous reaction sites is essential for the expression of resistance and is a T-cell and/or humoral factor dependent immune mechanism.

2.7 EXPRESSION OF RESISTANCE

Expression of resistance to tick infestation varies greatly depending on the species of tick and host concerned. In general the resistance is manifested by physical removal of the parasite, protracted feeding, failure to lay eggs and sometimes death *in situ* which could be the result of the same or different immune mechanisms.

In the *B. microplus*-cattle system the resistance is manifested by the rejection of larvae in the first 24 hours of the feeding cycle, without any apparent damage to the ticks (Roberts, 1968; Roberts, 1971), reduction in engorgement weight (Wagland, 1978) prolongation of feeding time, reduction in egg laying and viability (Hewetson, 1971) and death of the parasite *in situ* (Riek, 1962).

However, Bennett (1969) demonstrated that physical restraint from grooming in resistant hosts resulted in a significant increase in tick yield. This suggests that the major expression of resistance is the physical removal of the parasite rather than its destruction (Willadsen, 1980).

Guinea-pigs and rabbits acquire resistance to ticks after one infestation (Trager, 1939a; Allen, 1973; Bagnall, 1978; Wikel and Allen, 1976a; Bowessidjaou *et al.*, 1977; Brown and Knap, 1981). Resistance was expressed by allowing fewer ticks to engorge and by reduced weight of the blood meal. Resistance to *D. andersoni* larvae (Allen, 1973) was manifested by a reduction to 3-6% in the percentage of larvae engorging compared with 80% during primary infestation. Similarly, challenge infestations of *A. americanum* and *R. sanguineus* resulted in 58% and 62% tick rejection respectively (Brown and

Askenase, 1981). Exposure of sensitized guinea-pigs to R. appendiculatus and I. holocyclus larvae resulted in greater than 90% tick rejection in most of the guinea-pigs (Askenase et al., 1982). Further, the level and duration of resistance was seen to be influenced by the strength and size of the initial sensitizing dose. In another study Bagnall and Rothwell (1974) found that a challenge infestation of I. holocyclus resulted in the majority of the larvae dying in situ, thus reducing the numbers of engorging ticks to 2-3%, as compared to 40-60% following primary infestation.

Acquisition of resistance to I. ricinus in rabbits was expressed by an increase in the duration of feeding time, decreased weight of the blood meal and reduced egg production (Bowessidjaou et al., 1977). Feeding time increased from 172 hours on primary infestation to 220 hours at the fourth infestation. In addition 36% of the ticks died in the host without engorging. Similar observations on R. appendiculatus were made by Branagan (1974).

All these studies suggest that the major expression of resistance is rejection of the parasite achieved by various hypersensitivity reactions, but how these immunologic reactions affect the tick is still not clear.

CHAPTER THREE

GENERAL MATERIAL AND METHODS

CONTENTS

3.1 TICKS

3.1.1 Origin

Ticks used in this study were from the H. a. anatolicum colony maintained at the Centre for Tropical Veterinary Medicine, originally supplied by Professor B.S. Gill from a colony maintained at the Punjab Agricultural University, Ludhiana, India.

3.1.2 Storage

Unfed and engorged ticks of all instars were stored in plastic tubes with ventilated lids. Ovipositing females and engorged larvae and nymphs were maintained at a constant temperature of 23°C and 85% relative humidity (over a saturated solution of potassium chloride) throughout egg laying/moulting and post moulting development. After the completion of post-moult development ticks were stored at 18°C and 85% relative humidity and were used when two to five months old.

3.1.3 Feeding

For feeding ticks, the methods described by Hosie (1978) were followed. Depending upon the requirements H. a. anatolicum larvae were fed on rabbits on which they behaved as two host ticks.

For feeding ticks on rabbits both the body and the ears were used as attachment sites. Twenty-four hours prior to application of ticks, the hair from the ears or back and flanks of the rabbit were clipped closely with electric small animal clippers and the feeding sites surface sterilized with 70% alcohol. Ticks were

restrained on the body or ears in bags of thick cotton (ear/body bags). The body bags were kept tight with cuffs of soft elastic at both ends. The ear bags were bound around the base of the ears with strips of zinc oxide adhesive tape. The bags were closed at their distal ends with rubber bands and tied to plastic collars which were used to restrain the rabbits from dislodging the ear/body bags (Bailey, 1960). The hind legs were also hobbled with zinc oxide tape around the hind feet to prevent scratching of the body bag.

For feeding ticks on calves the hair from the ears was removed and the ears washed with soap one day before application of ticks. Just before putting on the ear bags, the skin around the base of the ears was de-greased with a mixture of xylene and alcohol, and a strip of zinc oxide adhesive tape fastened around the base of the ear. The ear bags were then slipped over the ears and their bases secured in position with adhesive tape. The bags were closed at the distal ends.

3.2 EXPERIMENTAL ANIMALS

3.2.1 Rabbits

Female New Zealand white rabbits two to three months old, supplied by Edinburgh University, Centre for Laboratory Animals, Easter Bush, having no previous contact with ticks, were used. They were confined individually in ordinary cages in an isolation room in the small animal house at C.T.V.M. They were fed a standard pelleted diet and hay.

3.2.2 Cattle

Bos taurus (Ayrshire or Ayrshire x short horn) four to five month old calves weighing 60 to 75 kg, were supplied from local farms and the likelihood of their previous exposure to ticks was minimal. During experiments they were housed in the tick isolation unit without special confinement at 20°C and 65 to 95% relative humidity. They were fed on hay and calf weaner pellets.

3.3 DISSECTION OF SALIVARY GLANDS

Ticks of either sex were immobilised with their dorsal surfaces upward in paraffin wax in a Petri dish. The ticks were then covered with a shallow layer of cold saline (0.85%). Using a fine scalpel blade and fine forceps, the dorsal integument was removed under a stereoscopic dissecting microscope. The gut diverticula covering the salivary glands were removed and the glands dissected free of the tracheae and other tissues. The salivary glands were then removed after severing the main ducts at their anterior ends.

3.4 BIOPSY PROCEDURE

Biopsies of tick feeding sites on the ears of calves were carried out as follows. Twenty minutes in advance the animal was injected with a 2% solution of the sedative Rompun (Bayer) intramuscularly at a dose of 0.4 ml/50 kg body weight. Once the calves were sedated to recumbency the area around the tick feeding sites were infused with local anaesthetic (2% Xylocaine) and feeding sites with attached mouthparts were biopsied using a 3.5 mm trephine

which rotated rapidly with a hand-held electric motor. Biopsies were immediately placed in Karnovsky's fixative at 4°C.

3.5 PROCESSING OF MATERIALS FOR HISTOLOGICAL, HISTOCHEMICAL, HISTOPATHOLOGICAL AND ULTRASTRUCTURAL EXAMINATION

3.5.1 Light Microscopy Studies

3.5.1.1 Methacrylate sections:

(i) Salivary glands: Groups of three or four salivary glands were fixed in Karnovsky's fixative (Appendix 1.1) in 0.15M phosphate buffer, pH 7.4 at 4°C for one hour and then embedded in methacrylate plastic (hydroxyethyl methacrylate or GMA) (Taab) using the method of Bennet et al. (1976) with some modifications. After fixation the salivary glands were rinsed three times, each for ten minutes in distilled water. This was followed by dehydration through 75%, 90% and 100% methacrylate (Appendix 1.2) in distilled water, 30 minutes each. Once dehydrated the salivary glands were infiltrated with a mixture of methacrylate and benzyl peroxide (BPO) (Polaron Equipment Ltd.)/polyethylene glycol (PEG) (Taab) (4.7 ml + 0.3 ml respectively, Appendix 1.3), three changes of 30 minutes each. After the third infiltration the salivary glands were embedded in a freshly prepared mixture of GMA, PEG/BPO and dimethylaniline (DMA) (4.7 + 0.3 ml + 0.5 ml) over ice in plastic capsules (1 ml and 3-4 salivary glands per capsule). Polymerization was carried out at 4°C overnight and then at 25-35°C for a further 72 hours.

(ii) Tick feeding sites: Tick feeding sites with attached mouthparts (4 x 4 mm) were fixed in Karnovsky's fixative (containing

0.025% calcium chloride) for four hours at 4°C. After primary fixation the tissues were trimmed to 3 x 3 mm size and again immersed in fresh fixative overnight. After fixation the tissues were washed in distilled water (3 x 30 minutes) and dehydrated through 75%, 90% and 100% methacrylate (one hour each) and then left in 100% methacrylate overnight. Once dehydrated the tissues were infiltrated with a mixture of GMA and BPO/PEG (4 x 2 hours), left overnight in the last infiltrate mixture. They were then embedded and polymerized in the same way as the staining glands.

Semi-thin sections (1-2 µm) were cut with glass knives on a Cambridge Huxley ultramicrotome (Cambridge Science Instruments Ltd.).

3.5.1.2 Cryostat sections: Tick feeding sites with attached mouthparts were snap-frozen in liquid nitrogen and used when required. Five micron sections were obtained in a Slee type HS cryostat. The sections were allowed to dry for 30 to 60 minutes at room temperature and fixed or directly stained/incubated for histochemical methods, as described in Bancroft (1975).

3.5.1.3 Paraffin sections: Tissues were fixed in Karnovsky's fixative containing 0.025% calcium chloride for 16 hours, rinsed in distilled water, dehydrated through a graded ethanol series, cleared in "Histoclear" (National Diagnostic) and embedded in "fibre-wax" (Raymond A Lamb). Five micron thick sections were cut on a rotary microtome (Leitz Wetzlar), deparaffinized, hydrated and stained as required.

3.5.1.4 Whole salivary glands: Whole salivary glands (WSG) were fixed or directly stained/incubated in medium containing the substrate specific for the enzyme tested as detailed in Bancroft (1975). WSG were also processed for GMA embedding after reactions for enzymatic localization were complete.

3.5.1.5 Staining:

(i) Giemsa stain: For histological examination of salivary glands, methacrylate sections were stained with 10% Giemsa stain, pH 7.2 for an hour. After staining the sections were rinsed briefly in water, differentiated in 20% methanol, air dried and mounted in DPX (Raymond A Lamb).

For histopathological examination of tick feeding sites, methacrylate sections (1-2 µm) were stained with 10% acidic Giemsa stain (Giemsa stain diluted in 0.05M acetate buffer, pH 4.5-5.0) for an hour and then differentiated in aqueous buffer (pH 7.4) for ten minutes. The stained sections were then air dried and mounted in DPX. Paraffin sections were stained for an hour in acidic Giemsa stain as above, dehydrated through ascending series of alcohols, cleared in xylene and mounted in DPX.

(ii) Collagen staining: For staining collagen Massons trichrome technique (Masson, 1929) and Van Gieson's method (1889) described in Bancroft and Stevens (1982) were followed. Control tissues were treated with collagenase (1%) at 37°C for three hours before staining for collagen.

3.5.1.6 Quantification of cellular responses: In order to quantify the participation and arrival order of various inflammatory cells in the feeding lesions, detailed cell counts were performed. All the cells encountered in 20 oil immersion fields (x 100) around the mouthparts in sections stained with acidic Giemsa stain were counted. Figure 3.1 shows this pattern.

Basophils were identified as polymorphonuclear leucocytes with prominent, metachromatic granules and were readily distinguished from tissue mast-cells which had large unilobed nuclei, with numerous small metachromatic granules and often with — — dendrite-like processes. Eosinophils had bilobed nuclei and numerous large sausage shaped, bright red staining granules. Neutrophils had multilobed nuclei and small bright red granules. Considerable difficulty was experienced initially in differentiating eosinophils from neutrophils in sections from rabbits, as the granules of both leucocytes stained bright red. This difficulty was overcome with experience and careful observations.

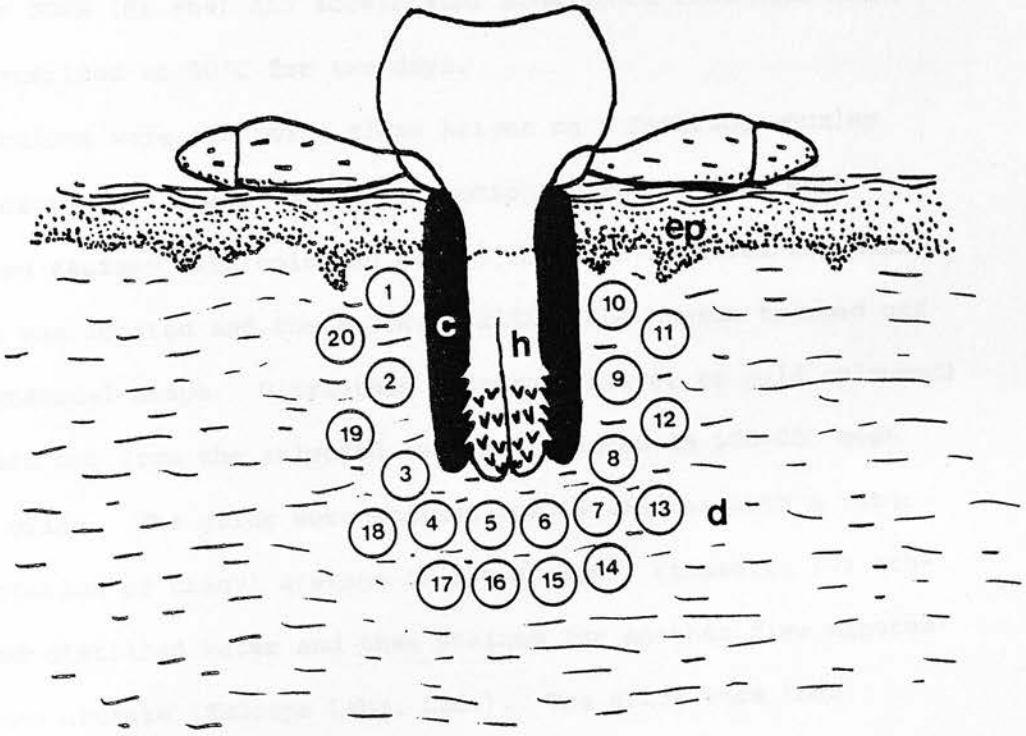
In cattle the neutrophil granules were unstained or very weakly stained, whereas eosinophil granules stained bright red and were very easy to identify.

3.5.2 Electron Microscope Studies

Salivary glands were fixed in 2.5% glutaraldehyde (Taab) in 0.1M cacodylate buffer pH 7.4 at 4°C for two hours, washed in the same buffer (3 x 10 minutes), post-fixed in 1% Osmium tetroxide (OSO_4) (Johnson Matthey Chemicals Ltd.) in cacodylate buffer at room temperature, dehydrated through ascending grades of ethanol

Figure 3.1 Diagrammatic representation of a tick feeding site showing the location of fields (1-20) examined for the quantification of the cellular responses.

c = cement cone; d = dermis; ep = epidermis; h = hypostome.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(2 x 15 minutes in each of 10%, 50%, 70%, 90% and 100% ethanol) and cleared in (2 x 15 minutes) prophyline oxide (Fisons). The prophyline oxide was replaced by a mixture (1:1) of propylene oxide/araldite CY 212 (Agar Aids) for one hour. The specimens were then allowed to infiltrate in pure araldite overnight (16 hours). The tissues were then embedded in fresh araldite with hardener DDSA (HY 964) and accelerator BDMA (both from Agar Aids) and polymerized at 60°C for two days.

Sections were cut using glass knives on a Cambridge-Huxley ultramicrotome. First, semi-thin sections were cut from each block and stained with toludine blue. The area required for examination was located and the excess araldite and tissue trimmed off in a pyramidal shape. Ultra-thin sections (silver to gold coloured) were then cut from the selected area and mounted on 100-200 mesh copper grids. The grids were stained for 15 minutes with a saturated solution of uranyl acetate in 50% ethanol, rinsed in 10% ethanol and distilled water and then stained for another five minutes with lead citrate (EMScope Labs. Ltd.). The grids were then rinsed in distilled water, dried and examined in a Philips 400 electron microscope.

3.6 HISTOCHEMICAL METHODS

The histochemical methods used for identification of proteins, polysaccharides, nucleic acids, lipids and various enzymes were based on those of Chayen et al. (1969), Humason (1972), Pearse (1972) and Bancroft (1975). Appropriate controls were included for each test (Tables 5.1 and 5.2).

3.6.1 Proteins

For identification of proteins, the mercuric bromophenol blue method of Mazia et al. (1953) as described in Humason (1972) was used. Methacrylate sections were immersed in absolute alcohol for one to two minutes before staining in a solution of mercuric chloride, bromophenol blue and 95% ethyl alcohol (10 g + 100 g + 100 ml, respectively) for 30 minutes. Sections were then washed in 0.5% aqueous acetic acid for 20 minutes, rinsed in tap water to convert the dye to the blue alkaline form, dehydrated by transferring directly to tertiary butyl alcohol, cleared in xylene and mounted in DPX.

Millons reactions (Baker, 1956) the diazotization coupling method (Glenner and Lillie, 1959) and the DMAB-nitrite method of Adams (1957) as detailed in Bancroft (1975) were used to identify tyrosine-containing proteins. For the detection of disulphides the performic acid-alcian blue method of Adams and Sloper (1955, 1956) and the DDD reaction of Barnett and Seligman (1952, 1954) were used. The DDD reaction (Barnett and Seligman, 1952, 1953/54) and mercury orange method of Bennett and Watts (1958) as detailed in Bancroft (1975) were used for the localization of sulphydryl groups. For methacrylate sections the period of staining was increased by 1.5 times as compared with cryostat sections/WSG.

3.6.2 Polysaccharides/Polysaccharide-protein Complexes

To locate polysaccharide groupings, the periodic acid-Schiff's reaction (PAS) as detailed in Bancroft (1975) was followed.

Sections were placed in a 1% aqueous solution of periodic acid for ten minutes, washed in tap water and distilled water, and treated with schiffs reagent (Appendix 1.4) for 15 to 20 minutes. The sections were then washed in tap water for ten minutes, counter-stained in haematoxylin for 15 minutes, washed in tap water, dehydrated through ethanol series to xylene and mounted in DPX. Polysaccharide activity was indicated by bright magenta staining. Components staining for both polysaccharide and proteins were assumed to be glycoproteins.

In order to differentiate various PAS positive materials, test sections and control sections were subjected to different extraction procedures. The loss of PAS staining following pre-incubation with 1% diastase in 0.02M phosphate buffer (pH 6) at 37°C for two hours was taken as an indication of the presence of glycogen. The presence of glycogen material was also confirmed by Best's carmine method (Best, 1905) as described in Bancroft (1975). Similarly, the loss of PAS staining following pre-incubation with a 1% aqueous solution of pectinase for two hours at 37°C was taken to indicate the presence of galactogen material. For detection of muco- and glycoproteins the sections were pre-treated with a solution of crystalline pepsin in 0.02N HCl (5 mg/ml) at 37°C for two hours. Loss of staining on PAS reaction was indicative of the presence of the cited complexes.

The alcian blue method of Steedman (1950), the azure A method of Hughesdon (1949) and the dialysed iron method of Müller (1955) and Mowery (1958) as described in Bancroft were followed for the detection of acid mucosubstances.

For demonstration of nucleic acids, the methyl green pyronin method (Kurnick, 1955) as detailed in Bancroft was followed.

3.6.3 Lipids

For identifying lipid material the oil red O method of Lillie and Ashburn (1943) and sudan black B method of Lison and Dagnelie (1935) as given in Bancroft were followed. For methacrylate sections the staining time was increased by 1.5 times. Control preparations were treated with a methanol-chloroform solution at 60°C for three to six hours (Pearse, 1972) prior to staining with oil red O or sudan black B.

3.6.4 Enzymes

3.6.4.1 Non-specific esterase: The alpha naphthyl acetate method of Gomori (1950) and indoxyl acetate method of Holt (1954) as detailed in Bancroft (1975) were followed.

For Gomori's method alpha naphthyl acetate (Sigma) was used as the substrate with fast blue B (Sigma) as the coupling agent. WSG, cryostat sections and methacrylate sections were incubated in substrate medium (Appendix 1.5.1) at room temperature for 15 minutes (WSG and cryostat sections) and 45 minutes (methacrylate sections). After incubation the WSG and the sections were washed in distilled water and mounted in polyvinylpyrrolidone (PVP) or dehydrated through graded alcohols to xylene and mounted in DPX. The esterase activity was indicated by reddish brown deposits. Control preparations were incubated as for the test minus the substrate.

For the indoxyl acetate method the incubating medium consisted of 5-bromo-4-chloro-indoxyl acetate (Sigma), potassium ferrocyanide, potassium ferricyanide, calcium chloride and tris buffer, pH 7.2 (Appendix 1.5.2). The tissues were incubated at 37°C for 45 to 60 minutes (WSG and cryostat sections) or two to three hours (methacrylate sections). After incubation the preparations were rinsed in tap water, counterstained in 1% neutral red, rinsed in tap water, dehydrated, cleared in xylene and mounted in DPX. Esterase activity was indicated by blue staining.

3.6.4.2 Aminopeptidase: The method of Nachlas et al. (1957) as given in Bancroft (1975) was used. The incubating medium (Appendix 1.6) contained L-leucyl-4-methoxy beta-naphthylamide (Sigma) as the substrate and fast blue B (Sigma) as the diazonium salt. Incubation was carried out at 37°C (30 minutes for WSG and crystat sections and one hour for methacrylate sections). After incubation the WSG and the tissue sections were rinsed in 0.85% sodium chloride, immersed in a 0.1M solution of copper sulphate (5 minutes), rinsed again in saline, dehydrated through graded alcohols to xylene and mounted in DPX. Aminopeptidase activity was indicated by red/purple staining. Control preparations were pre-treated with absolute ethanol at 37°C for 30 to 45 minutes before incubation in the test medium (Chayen et al., 1969).

3.6.4.3 Beta-glucuronidase: The post-coupling method of Fishman and Goldman (1965) as described in Chayen et al. (1969) was used. Naphthol AS-B1-beta-D-glucosiduronic acid (Sigma) was used

as the substrate with fast garnet GBC (Sigma) as the diazonium coupling agent. Preparations were incubated at 37°C for 30 minutes (WSG and cryostat sections) and one hour (methacrylate sections) in the substrate medium (Appendix 1.7). After incubation WSG and sections were washed in cold distilled water, immersed in a cold saturated solution of fast garnet GBC in 0.01M phosphate buffer, pH 7.4, washed in distilled water and mounted in PVP. Beta-glucuronidase activity was denoted by red staining.

3.6.4.4 Arysulphatases: The naphthol AS sulphate method given in Pearse (1972) was used. Naphthol AS-BI sulphate (potassium salt) (Sigma) was used as the substrate with pararosanilin hydrochloride (Sigma) as the diazonium coupling agent. Preparations were incubated for one hour (WSG and cryostat sections) and two hours (methacrylate sections) at 37°C in the substrate medium (Appendix 1.8). WSG and sections were washed in distilled water, counterstained with 2% methyl green, washed and mounted in PVP. Arysulphatase activity was indicated by red and brown deposits.

3.6.4.5 Phosphatases:

Alkaline phosphatase: The azo dye coupling method and the Gomori calcium method as detailed in Bancroft (1975) were used for the demonstration of alkaline phosphatase activity. For the azo dye coupling method sodium-alpha-naphthyl phosphate (Sigma) was used as the substrate. Cryostat sections or WSG and methacrylate sections were incubated in the working solution (Appendix 1.9.1) for one hour or two hours respectively at room temperature.

The preparations were then washed, counterstaind with methyl green, washed and mounted in PVP or quickly dehydrated in absolute ethanol, cleared in xylene and mounted in DPX. Alkaline phosphatase activity was denoted by reddish brown precipitates/staining.

For the Gomori calcium method sodium beta-glycerophosphate (BDH) was used as the substrate. WSG or cryostat sections and methacrylate sections were incubated in the working solution (Appendix 1.9.2) for one or 6-8 hours respectively at 37°C. The preparations were then washed, treated with 2% cobalt nitrate (5 or 10 minutes, respectively), washed, immersed in 1% ammonium sulphide (2 or 5 minutes, respectively), washed, counterstained, washed and mounted in PVP. Alkaline phosphatase activity was denoted by brownish black staining.

Acid phosphatase: The azo dye coupling method and Gomori lead methods as described in Bancroft (1975) were followed. The substrate used for the azo dye coupling method was sodium alpha-naphthyl phosphate (Sigma) dissolved in 0.1M veronal acetate buffer at pH 5.0 (Appendix 1.9.3). Incubation, washing, counterstaining and mounting procedures were the same as used for alkaline phosphatase.

For Gomori's lead method the incubation medium contained sodium beta-glycerophosphate as the substrate and lead nitrate (Appendix 1.9.4). WSG and cryostat sections were incubated in the working solution for two hours and methacrylate sections for four hours at 37°C. The preparations were immersed in 1% ammonium sulphide (fresh), washed, counterstained, washed and mounted in PVP. The acid phosphatase activity was denoted by black precipitates.

Adenosine triphosphatase: The lead method of Wachstein and Meisel (1960) as described in Bancroft (1975) was followed. WSG or cryostat sections and methacrylate sections were incubated at 37°C (for 30 to 60 minutes or 2 hours respectively) in a working solution containing 0.125% adenosine triphosphate (disodium salt) (Sigma) with 2% lead nitrate and 2.5% magnesium nitrate in a Tris buffer pH 7.2 (Appendix 1.9.5). Preparations were then washed in distilled water, immersed in 1% ammonium sulphide for two to five minutes, washed in tap water and mounted in PVP. The ATP-ase activity was denoted by brownish black deposits.

Glucose-6-phosphatase: The lead method of Wachstein and Meisel (1956) as detailed in Bancroft (1975) was followed. The potassium salt of glucose-6-phosphate (Sigma) was used as the substrate. Lead nitrate was included in the incubating medium (Appendix 1.9.6) to precipitate the phosphate produced during incubation. WSG and sections were incubated at 37°C for 20 minutes and methacrylate sections for 45 minutes. After incubation WSG and sections were treated as for ATP-ase. Glucose-6-phosphatase activity was indicated by brownish black deposits.

3.6.4.6 Oxidoreductases:

Cytochrome oxidase: The metal chelation method (Burstone, 1959) as described in Bancroft (1975) was followed. WSG or cryostat sections and methacrylate sections were incubated in the substrate medium (Appendix 1.10.1) containing 4-amino diphenylenediamine (Sigma) and 1-hydroxy-2-naphthoic acid (Koch-Light Laboratories Ltd.) at 37°C for 45 minutes or two hours respectively.

After incubation preparations were transferred directly to 1% cobalt acetate solution for one hour, washed in distilled water and mounted in PVP. Cytochrome oxidase activity was indicated by blue black deposits. Control preparations were treated with potassium cyanide as for DOPA-oxidase.

DOPA-oxidase (tyrosinase): The DOPA reaction (Becker et al., 1935) as described in Bancroft (1975) was used. D.L. 3:4-dihydroxyphenylalanine (Sigma) in 0.1M phosphate buffer (pH 7.4) was used as the substrate. WSG/cryostat sections and methacrylate sections were incubated at 37°C in the substrate medium (Appendix 1.10.2) for five hours or eight hours respectively. The preparations were then washed, counterstained with 2% methyl green, washed in distilled water and mounted in PVP. DOPA-oxidase activity was indicated by brownish black staining. Control preparations were incubated for 30 minutes in 10^{-3}M potassium cyanide (3.25 mg/50 ml) in 0.1M phosphate buffer, pH 7.4 at 37°C (Chayen et al., 1969) before incubation in the substrate medium.

Monoamine oxidase: The tetrazolium method of Glenner et al. (1957) as described in Bancroft (1975) was used. The substrate medium (Appendix 1.10.3) contained tryptamine hydrochloride (Sigma) as the substrate and tetranitro-blue (Sigma) as the tetrazolium salt. Preparations were incubated in the substrate medium at 37°C for 45 minutes (WSG and cryostat sections) and 1½ hours (methacrylate sections). After incubation WSG and tissue sections were washed in tap water, fixed/washed and mounted in PVP. Monoamine oxidase activity was indicated by blue-black deposits.

Succinate dehydrogenase: For succinate dehydrogenase demonstration the method of Pearse (1972) was followed. Preparations were incubated at 37°C for one hour (WSG and cryostat sections) and two hours (methacrylate sections) in a substrate medium (Appendix 1.10.4) containing sodium succinate (Sigma) and nitroblue tetrazolium (NBT) (Sigma). After incubation WSG and sections were washed, counterstained with 2% methyl green, washed and mounted in PVP. Succinate dehydrogenase activity was denoted by black deposits.

NADH diaphorase: The MTT method of Pearse (1972) was followed. Co-enzyme NADH (Sigma) was used as the substrate with 3(4.5-dimethylthiazolyl-2)5-diphenyl tetrazolium bromide (MTT) (Sigma) as the tetrazolium agent. Preparations were incubated in the substrate medium (Appendix 1.10.5) for 30 to 45 minutes (WSG and cryostat sections) or one hour (methacrylate sections) at 37°C. WSG and sections were washed in water, counterstained, washed and mounted in PVP. NADH diaphorase activity was indicated by black formazan deposits.

NADPH diaphorase: The method of Pearse (1972) was followed. The preparations were incubated at 37°C for 45 minutes (WSG and cryostat sections) or two hours (methacrylate sections) in the incubating medium containing co-enzyme nicotinamide adenine dinucleotide phosphate reduced (NADPH) (Sigma) as the substrate and NBT as the tetrazolium salt (Appendix 1.10.6). After incubation WSG and sections were post-fixed or washed in water, dehydrated through graded alcohols to xylene and mounted in DPX. NADPH diaphorase activity was denoted by purple formazan deposits.

3.6.5. Controls

Cryostat sections of liver, kidney, heart, duodenum, intestine and skin from rabbits were used as positive controls (Tables 5.1 and 5.2). Negative control preparations of salivary glands and tick feeding sites in each test were incubated in the test medium without substrate. Some tests also included specific control procedures as detailed in the test.

3.7 ISOLATION AND CHARACTERIZATION OF SALIVARY ANTIGENS

3.7.1 Salivary Gland Extracts (SGE)

The salivary glands were removed from unfed, 24, 96 and 144 hour fed female ticks and washed three times in ice cold distilled water. They were then homogenized by hand in a 1.0 ml capacity Griffiths tube glass homogenizer (20 pairs of salivary glands/1.0 ml distilled water) for two minutes over ice. The resulting preparation was centrifuged at 10,000 g for 45 minutes and the SGE (supernatant) collected and stored at -20°C until required. Protein concentrations of the extracts were determined by the method of Lowry *et al.* (1951).

3.7.2 Collection of Saliva

Adult female ticks (75 ± 20 mg body weight) which had fed for 96 hours were held on a specially made perspex platform with their dorsal surface upward using double-sided adhesive tape.

A haematocrit capillary tube was placed over the hypostome and chelicerae of each tick. The ticks were then injected with dopamine (3-hydroxytyramine hydrochloride) at a rate

of 20-30 µl/tick (1.5 mg/ml in 1.2% saline) into the haemolymph with a fine glass needle attached by fine tubing to a screw operated tuberculin syringe. The needle was withdrawn one minute after injection. The perspex platform with ticks was then removed from the microscope stage and placed in a humidified box at 23°C for 45 minutes to allow saliva secretion to take place. Only colourless saliva was collected. Saliva from 100 females was pooled together and its protein concentration determined by the method of Lowry et al. (1951).

3.7.3 Immune Serum

Immune serum was collected from a group of three rabbits, which had been infested three times at 28 day intervals with 20 female and 20 male ticks. At 144 hours after tertiary infestation 20 ml of blood was collected from the marginal ear vein of each rabbit. The blood was allowed to clot at room temperature for one hour, at 37°C for another hour and then at 4°C overnight. Serum was separated by centrifuging at 1500 g for 30 minutes and stored at -20°C until required.

3.7.4 Preparation of Samples for Sodium Dodecyl Sulphate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples of saliva and SGE were dissolved (1:1 v/v) in a reducing solution (18.05 mM Tris, 30 mM EDTA, 5% SDS, 2.5% mercaptoethanol, 25% glycerol and a trace of 0.25% bromophenol blue) and the protein concentration adjusted to 30 µg/100 µl. They were then heated for five minutes at 100°C, cooled and stored at -20°C until required.

3.7.5 SDS-PAGE

For determining the protein profiles, the SGE from unfed, 24, 96 and 144 hour fed female ticks, and saliva from 96 hour fed females, were separated by SDS-PAGE on 7% acrylamide gel slabs. Gels used comprised of a 3 cm stacking gel (total concentration of acrylamide + bisacrylamide (T) = 4.5%, concentration of bisacrylamide (C) = 2.6%, 0.1% SDS, 0.125 M Tris/HCl pH 6.8) and a 19 cm separating gel (T = 7%, C = 2.6%, 0.1% SDS, 0.375 M Tris/HCl pH 8.8). The electrophoresis buffer was 0.025 M Tris, 0.192 M glycine and 0.1% SDS, pH 8.3 (O'Farrel, 1975).

Samples equal to 30 µg of protein were applied in 100 µl volumes into sample wells. Electrophoresis was carried out at 70 volts until the bromophenol blue reached the end of the gel, usually 16 to 20 hours.

3.7.6 Detection of Protein Bands After Electrophoresis

Proteins were visualised by staining with 0.1% (w/v) Coomassie blue R-250 (Sigma) in destain solution (25% methanol; 10% acetic acid; 65% water) at room temperature for two to four hours. Gels were destained overnight in the destain solution with continuous shaking. Prior to photography, gels were immersed in distilled water for one hour.

For increased sensitivity, gels originally stained with Coomassie blue were first destained and then restained using Morrissey's (1981) method for silver staining as follows.

The destained gels were incubated in solution 1 (Appendix 1.11.1) for 15 minutes in a fume cupboard with occasional shaking.

The gels were washed in distilled water (3 x 20 minutes) and then incubated for another 20 minutes in solution II (Appendix 1.11.2) with occasional shaking. The gels were washed as before and incubated in solution III (Appendix 1.11.3) until the desired colour developed (5-10 minutes). The reaction was stopped with 5% acetic acid, the gels left in this for one hour and then in water. The gels were photographed soon after developing.

3.7.7 Calculation of Protein Molecular Weights

Protein molecular weights were calculated by reference to the mobilities of a mixture of low molecular weight calibration proteins which included phosphorylase (94,000 daltons), albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), trypsin inhibitor (20,100 daltons) and alpha-lactalbumin (14,400 daltons) (Pharmacia).

3.7.8 Concanavalin A - Peroxidase Method for Detecting Glycoproteins

Gels were washed overnight in isopropanol/glacial acetic acid/water (25:10:65, v/v), then overnight in isopropanol/glacial acetic acid/water (10:10:80, v/v) and finally overnight in glacial acetic acid (10%). The gels were then washed to pH 7.2 with phosphate buffered saline (PBS) (10 mM phosphate buffer pH 7.2 with 0.145 M sodium chloride). The washed gels were incubated with Concanavalin A (Con A) (0.5 mg/ml of Con A in PBS pH 7.2) for three hours after which unbound Con A was removed by washing overnight with PBS. Gels were then incubated with horseradish peroxidase (Sigma type II, 0.1 mg/ml in PBS) for three hours. Unbound peroxidase

was removed by washing with PBS overnight. Gels were then incubated with 3-3'diamino-benzidine tetrahydrochloride (Sigma grade II, 0.5 mg/ml) and hydrogen peroxide (0.5 µl, 100 volumes H_2O_2 per ml PBS). Brown-stained glycoprotein bands appeared within 10 minutes after which the reaction was stopped by washing the gel with 10% acetic acid.

3.7.9 Separation of Enzymes using Agarose Isoelectric Focusing (IEF)

Agarose gels 0.5 mm thick were cast using the LKB casting system. Gels were prepared using Isogel agarose (Miles Ltd.) (0.2 g), sucrose (3.6 g), tissue-culture-grade water (18 ml) and Pharmalyte of the appropriate pH intervals (1.2 ml). Gel running conditions and electrode buffer solutions were as detailed by Pharmacia. After focusing, gels were stained for the appropriate enzymes (Harris and Hopkinson, 1977).

3.7.10 Identification of Antigenic Proteins

Proteins from SGE and saliva from 96 hour fed female ticks were separated by SDS-PAGE on 7% gels. The protein bands were then electrophoretically transferred on to nitrocellulose paper at room temperature at 200 mA for five to six hours in blotting buffer (20 mM Tris/Glycine, pH 9.2 in 20% methanol). When the transfer was complete the track containing the molecular weight markers was cut off and stained with 1% amido black. The nitrocellulose paper (blot) was then placed into washing buffer (50 mM Tris, 105 mM NaCl, 1 mM EDTA, 0.05% NP_40, 0.25% gelatin and 0.2% azide) for two hours to block unreacted protein binding. The blots were then incubated

overnight with specific anti-tick immune serum (diluted 1:200 either in washing buffer when the second antibody was ^{125}I-labelled or in phosphate buffered saline containing 0.05% Tween-20 (PBST) when the second antibody was peroxidase conjugated). The blots were then carefully washed in washing buffer or PBST (6 x 8 hours) and then incubated with either ^{125}I-labelled goat anti-rabbit IgG or with horseradish peroxidase-conjugated goat anti-rabbit IgG (GARP) at a dilution of 1:1000 using a modification of Tsang et al. (1983). The blots were washed as before. The antigen-antibody complexes detected by incubation with GARP were then visualized by incubating with an appropriate substrate solution (50 mg of 3-3' diaminobenzidine tetrahydrochloride (Sigma grade II), 100 µl of H_2O_2 in PBS pH 7.2 (per 100 ml) for ten minutes. The stained blots were then rinsed thoroughly with water, dried and photographed.

Blots incubated with radio-labelled antibody were thoroughly dried prior to exposure for autoradiography at -79°C. FUJI-RX x-ray film and an intensifying screen were used with an overnight exposure. The x-ray film was then developed and photographed in the normal manner.

The following controls were included in this experiment:

(i) Normal serum instead of immune serum:

(ii) Omission of first antibody;

(iii) Omission of second antibody;

(iv) Omission of both the first and second antibodies.

3.7.11 Isolation of Antigenic Proteins from Saliva

Saliva (3 ml equal to 900 µg protein) was fractionated by SDS-PAGE on 7% acrylamide gel slabs. After electrophoresis a 2.5 cm wide track of the gel was cut off and stained with Coomassie blue to visualise the proteins. Using the stained gel as a template, protein bands corresponding to molecular weights of 130,000 (antigen I), 103,000 (antigen II) and 96,000 (antigen III) daltons respectively were cut from the unstained portion of the gel and transferred into 10 ml centrifuge tubes containing ice cold PBS (0.5 ml). The portions of polyacrylamide were macerated using a glass rod and the proteins were allowed to elute for 90 minutes. The tubes were then centrifuged at 10,000 g for 30 minutes, the supernatant removed and the protein contents estimated as before. The antigens thus isolated were kept at 4°C and inoculated into test rabbits within 30 minutes.

The three antigenic bands were selected because they formed the bulk of high molecular weight salivary proteins.

3.7.12 Skin Testing

The backs of rabbits (four hypersensitized and one naive control) were shaved 24 hours prior to skin testing. The skin was surface sterilized with 70% alcohol six hours prior to inoculation. Antigens I, II, III, whole saliva and SGE were administered intradermally at a concentration of 20 µg protein in 0.1 ml. Phosphate buffered saline and acrylamide in PBS were injected as controls. The diameter of the reactions were measured (in two directions at a right angle) at 15, 30, 60 minutes and then at 2, 4, 24 and 48 hours post inoculation. The mean of the two diameters was taken as the final observation.

CHAPTER FOUR

STRUCTURAL STUDIES ON THE SALIVARY GLANDS

CONTENTS

4.1 INTRODUCTION

The salivary glands of ixodid ticks are organs highly specialized for their diverse functions. During feeding they secrete a cement substance, that ensures attachment to the host for several days, anticoagulants, a variety of enzymes and other pharmacological agents which help in the development of the feeding lesion and provide an ample flow of blood (Sauer, 1977). They transform to excrete excess water to regulate the ionic balance of the haemolymph and help in concentrating the blood meal. The functions, other than those involved with feeding, include secretion of concentrated salts to absorb water from unsaturated air and secretion of proteinaceous material by males during copulation. In addition the salivary glands have been found to be the source of paralytic toxins (Ross, 1926; Balashov, 1972) and other components which stimulate allergic reactions in susceptible hosts. A knowledge of the functional morphology of the salivary glands is a necessary prequisite for an understanding of saliva production.

In recent years the salivary glands of ixodid ticks have been the subject of several histological (Till, 1961; Chinery, 1965; Balashov, 1972; Binnington, 1978) and ultrastructural studies (Kirkland, 1971; Coons and Roshdy, 1973; Meredith and Kaufman, 1973; Megaw and Beadle, 1979; Fawcett et al., 1981a,b; Krolak et al., 1982; Walker et al., 1984). These studies have shown that the salivary glands of ixodid ticks contain two morphologically distinct types of acinus: one agranular (type I) concerned with secretion of hygroscopic salts and the others (types II, III and IV) concerned with the secretion of granular material. The type III acinus

transforms during feeding to form the water excretory unit.

The present study aimed to examine the sequence of morphological and ultrastructural changes occurring in the salivary glands of both male and female H. a. anatolicum ticks during feeding.

4.2 EXPERIMENTAL DESIGN

The salivary glands from at least 10 to 15 ticks (10 ticks for ultramicroscopy and 15 ticks for light microscopy) of each sex, each from unfed, 24, 72 and 120 to 144 hours fed ticks were examined by light and electron microscopy. For light microscopy the salivary glands were fixed in cold Karnovsky's fixative for one hour and then embedded in methacrylate plastic. Semi-thin sections stained with alkaline Giemsa's stain, were examined for morphological details.

For ultrastructural examination the salivary glands were fixed overnight at 4°C in 2.5% glutaraldehyde, post-fixed for two hours in 1% osmium tetroxide and embedded in araldite. Ultra-thin sections stained with uranyl acetate and lead citrate were examined on a Philips 400 electron microscope.

4.3 RESULTS

The paired salivary glands of H. a. anatolicum were situated in the anteriolateral region of the body cavity. Each gland consisted of three morphologically distinct types of acinus in females (acinus types I, II and III; Figures 4.1-4.3) and four types in males (acinus types I, II, III and IV; Figure 4.4). The main salivary duct extended posteriorly and branched into several secondary and numerous tertiary ducts. The acinus types were distributed all along the duct system giving the appearance of a bunch of grapes.

4.3.1 Salivary Ducts

The salivary duct consisted of a layer of flattened epithelial cells which rested on a basement membrane and a thick cuticle towards the apical surface. The cuticle layer facing the duct lumen extended into the lumen of acini of types II, III and IV forming a valve and into the central cell of type I acini forming a nipple-like structure.

4.3.2 Type I Acini

The type I acini were found in the anterior region of the gland attached directly to the main duct through a short lobular duct. These acini were non-granular and lacked a valve-like structure. They consisted of four distinct types of cells: the peripheral cells, central cell, constrictor cell and neck cell (Figure 4.5). Each peripheral cell had two morphologically distinct regions: the basal region with numerous membraneous infoldings, and an apical region without infoldings (Figure 4.6). The apical region had electron lucent cytoplasm with a nucleus, numerous microtubules, lysosomes and sparse mitochondria. The apical membranes of these cells were closely applied against the plasma membranes of the central cell. However, no types of specialized junctions were seen.

The central cell had moderately electron dense cytoplasm with a large nucleus, numerous microtubules, mitochondria, several Golgi bodies (Figure 4.10) and many free ribosomes. The RER was poorly developed. The apical region of the central cell formed a specialized border with the single constrictor cell in the form of a desmosome-like junction having numerous microtubular structures

protruding from the borders into the cytoplasm of the cells (Figures 4.7 and 4.8). At the peripheral end the plasma membranes of the central cell formed a basal labyrinth by interdigitating with the basal infoldings of the peripheral cells (Figures 4.5 and 4.6). Associated closely with these basal infoldings were a large number of long irregularly shaped mitochondria (Figures 4.5, 4.6 and 4.9). The central cell communicated with the main salivary duct through a short cuticle-lined lobular duct which formed a nipple-like structure at its opening (Figures 4.7 and 4.8).

A single ring shaped cell, the constrictor cell (Krolak et al., 1982) was present anterior to the central cell. It was through this cell that the central cell communicated with the main salivary duct. It had electron lucent cytoplasm with numerous microtubular structures and a few mitochondria (Figures 4.5 and 4.7). Several nerve axons were seen closely associated with the apical membranes of peripheral cells (Figures 4.5 and 4.7). Surrounding the acinar duct at the hilus of the acinus were four small neck cells, with sparse cytoplasmic organelles (Figure 4.5).

In the unfed tick the cytoplasm of the central and peripheral cells had numerous lipid-like droplets and glycogen-like material (Figure 4.9). During feeding the type I acini did not exhibit any morphological changes apart from the disappearance of lipid and glycogen material (Figure 4.5). In addition the Golgi and myelin bodies became more common and occasionally a few electron dense granules of different sizes were also seen in the cytoplasm (Figures 4.6 and 4.10).

4.3.3 Type II Acini

The type II acini consisted of five distinct granular cell types (a, b, c_1, c_2 and c_3) arranged in a concentric manner around a small lumen. The acinal lumen communicated with the main branches of the salivary gland duct system through a cuticle-lined lobular duct, the opening of which was guarded in the lumen by a bicuspid valve. The granular cells were separated from each other by interstitial cells.

a cells: There were two a cells confined to the hilus of the acinus on either side of the valvular duct. In both male and female unfed ticks, they occupied the bulk of the acinus and were packed with numerous complex eosinophilic granules (Figure 4.11). These granules were composed of a number of electron lucent sub-units bound together in an amorphous electron dense matrix (Figures 4.11 and 4.15). Interspersed around these electron lucent sub-units were several electron dense rods (Figure 4.15). The cytoplasmic organelles included distinct RER, numerous free ribosomes, mitochondria and occasional Golgi bodies (Figure 4.15). The a cells lost most of their secretory granules during early feeding. However, small shrunken cells could still be seen during the middle period of feeding (Figure 4.12) and had almost disappeared towards the end.

b cells (capped granules): One or two b cells were present adjacent to a cells (Figures 4.11 and 4.12). In the unfed ticks these cells had numerous loosely packed large sized granules which stained greyish to light blue with Giemsa's stain (Figure 4.2). The granules were electron dense with distinct semi-circular caps of medium density (Figures 4.12, 4.13 and 4.16). The visibility of

capped structures depended upon the plane of the section. The cytoplasm of b cells had abundant RER, mostly running in parallel arrays, free ribosomes and moderate numbers of well developed mitochondria. At the onset of feeding there was an overall increase in the size of the cells and the numbers of their secretory granules (Figures 4.12 and 4.13).

c_1 cells: There were four medium sized c_1 cells confined to the fundus of the acinus. The granules of these cells were small to medium sized and stained different shades of blue with Giemsa's stain (Figure 4.2). Along with other cell organelles the cytoplasm had very well developed RER with moderately distended cisternae (Figures 4.17-4.20). The granules varied in their electron densities from moderate to dense (Figures 4.17-4.19). During feeding the cells increased enormously in size and were packed with secretory granules in their apical regions. Around 72 hours of feeding a number of distinct Golgi bodies were seen in the cytoplasm (Figures 4.20 and 4.21). Granules of slight, moderate and marked density were seen in the Golgi regions (Figure 4.21).

c_2 cells: There were at least two c_2 cells of medium size, situated next to a or b cells (Figures 4.11 and 4.12). In unfed ticks these cells were loosely packed with medium to large sized electron dense granules with irregular margins (Figures 4.11-4.13). The granules stained strongly purple with Giemsa's stain (Figure 4.2). The cytoplasm of c_2 cells had a considerable amount of RER with many free ribosomes. Moderate numbers of well developed mitochondria were distributed throughout the cytoplasm (Figures 4.22 and 4.24). The nucleus was large and heterochromatic. During feeding there was an

enormous increase in the size and the granular activity of the cell, with RER becoming more distinct (Figures 4.12 and 4.24).

c_3 cells: There were two of these cells in females and four in males. These were packed with secretory granules in unfed ticks (Figure 4.11). The granules were large in size and stained clear to light blue with Giemsa's stain. Their electron densities varied within the cell from light to moderately dense but were always less dense than c_1 granules (Figures 4.14, 4.25 and 4.26). The RER was well developed and rich in ribosomes. A large number of mitochondria were seen uniformly distributed in the cytoplasm (Figure 4.25). Golgi bodies were occasionally seen. In the females most of the granules were secreted during early feeding, but in males the granular activity remained constant throughout feeding.

4.3.4 Type III Acini

These were found to be distributed in the distal region of the salivary glands. Each acini consisted of three granular cell types: d, e and f arranged around a common lumen similar to that in type II acini (Figures 4.1 and 4.27). In unfed ticks the lumen of the acinus was small and contained a mass of microvilli compressed together along the centre of the acinus. However, it enlarged and became more obvious as feeding progressed (Figure 4.3).

d cells: There was one d cell present at the hilus of the acinus and it was similar in structure and location to the a cells of acinus II (Figure 4.27). In unfed ticks it occupied most of the acinar volume and was packed with complex secretory granules. The cytoplasm of the d cell was rich in RER and free ribosomes, and also

had a moderate number of medium sized mitochondria. A heterochromatic nucleus was seen in the basal region of the cell and was often indented by the granules. In female ticks most of the granules were lost during early feeding as in the a cells of type II acini.

e cells: There were four medium sized cells occupying the regions between the d and f cells (Figure 4.27). These cells were readily identified by their large eosinophilic granules (Figure 4.1). The cytoplasm of these cells was rich in RER, ribosomes and mitochondria (Figure 4.28). The granules were moderately electron dense and had a characteristic substructure of closely packed, moderately electron dense sub-units, giving a honeycomb-like appearance (Figure 4.29). Like a and d cells these cells had lost most of their secretory granules by 72 hours of feeding in female ticks. Towards the final stages of feeding these cells had almost disappeared and if present were seen to be compressed between the well developed interstitial cells (Figures 4.32 and 4.33).

f cells: These were a group of six relatively small cells confined to the fundus of the acinus (Figures 4.1 and 4.27). In unfed ticks these cells were agranular and had a large heterochromatic nucleus surrounded by moderately electron dense cytoplasm. At the onset of feeding the f cells became hypertrophied and had considerably increased in size by 72 hours of feeding. The secretory granules were small in size and moderately electron dense. The RER was well developed, often in parallel arrays and had numerous ribosomes (Figure 4.30). By 72 hours of feeding, the f cells underwent a rapid transformation in their external configuration. They moved away from the

basal lamina and formed the bulk of the acinar lumen by extending their microvillate surfaces along the lumen alternating with the adlumenal interstitial cells. By 120 hours, the nucleus of the f cells was left with a narrow rim of cytoplasm with numerous basolateral membranous extensions projecting into the intercellular spaces and interdigitating with the infoldings of ablumenal interstitial cells forming a labyrinthine structure (Figures 4.33-4.35).

The type III acini in males did not exhibit any appreciable changes in their morphology during feeding. Rather, there appeared to be a slight increase in the granular activity of the d and e cells. However, towards the later stages of feeding a small lumen was seen with interstitial cells becoming more prominent than before (Figure 4.31).

4.3.5 Type IV Acini

They were found distributed amongst type III acini in the distal regions of the glands of male ticks. These acini consisted of at least 12 g cells arranged in a concentric manner around an inconspicuous lumen and separated from each other by interstitial cells (Figure 4.37). In the unfed ticks these acini were seen as a group of nuclei with very little agranular cytoplasm. At the onset of feeding there was a progressive increase in the size of the acini accompanied by a massive accumulation of complex granules. These granules did not stain with Giemsa's stain. Towards the final stages of feeding these acini were found densely packed with irregularly shaped electron dense granules which contained several less dense areas (Figures 4.37 and 4.38). These granules were usually

seen to be indented against each other. The cytoplasm of g cells was rich in RER, usually concentrated in the basal region with moderated numbers of mitochondria. The nucleus was situated basally along the basal lamina (Figures 4.37 and 4.38). Golgi bodies with condensing vacuoles at their terminals were occasionally seen.

4.3.6 Interstitial Cells

All the granular cells of types II, III and IV acini were separated from each other by interstitial cells. In unfed ticks these cells were inconspicuous. They were present in two tiers: the adlumenal interstitial cells bordering the lumen and ablumenal interstitial cells (Fawcett et al., 1981b) having no direct access to this acinar lumen (Figures 4.14, 4.33-4.35). A single adlumenal interstitial cell extended all along the lumenal border alternating with the microvillate surface of the granular cells (Figures 4.31 and 4.35). The apical plasma membrane of this cell formed numerous microvilli which projected into the lumen. The lateral membranes joined with the adjacent granular cells by forming desmosome junctions (Figures 4.23 and 4.48) and bordered peripherally with the ablumenal interstitial cells. Numerous gap junctions (Figure 4.36) were noticed between the membranes of these two interstitial cells. The electron lucent cytoplasm contained a medium sized nucleus, numerous microtubules and a few mitochondria. Associated with the peripheral membranes were nerve axons.

The ablumenal interstitial cells had a prominent nucleus situated basally and cytoplasmic strands extending through the narrow intercellular spaces formed by the granular cells from the basal lamina

to the adlumenal interstitial cells (Figures 4.14, 4.27 and 4.31). The cytoplasm was moderately electron dense with several mitochondria and sparse RER. At the onset of feeding these cells became more prominent and increased progressively in size. In feeding females the lateral and basal plasma membranes of the ablumenal interstitial cells of type III acini formed extensive infoldings making a complex network of canaliculi all along the basal lamina (Figures 4.33-4.35). These infoldings were closely associated with large mitochondria (Figures 4.34 and 4.35). The infoldings interdigitated with the baso-lateral membranous extensions of the adjacent f cells to form a complex extracellular labyrinth (Figures 4.34 and 4.35). In type II acini of both sexes and III and IV acini of males the membranes of ablumenal interstitial cells were found to be convoluted to a small extent.

4.3.7 Secretory Process

The process involved in the secretion of the granular contents of salivary glands of H. a. anatolicum has been demonstrated in a series of photomicrographs (Figures 4.39-4.48). The granular salivary acini appeared to discharge their contents into the acinar lumen by a process of exocytosis.

Before secretion the secretory granules moved up to the apical region to lie in close apposition to the apical plasma membranes of their respective cells (Figure 4.39). This was followed by the fusion of the granule membranes to the plasma membranes of the cell (Figure 4.40). The subsequent reorganisation of these fused membranes eventually led to the discharge of secretory granules or

their products (Figures 4.41-4.45 and 4.48). The whole process appeared to be happening so quickly that it was very difficult to demonstrate all the stages. There appeared to be a transformation of the granule contents, especially of the large ones before secretion (Figures 4.43 and 4.44).

In addition there was extensive fusion of the granule membranes with one-another to form intracellular channels of interconnected granules (Figures 4.46 and 4.47). These channels led to the apical surface of the cell and released their products into the acinar lumen by fusion or rupture of their apical plasma membranes (Figures 4.46 and 4.47). Granules with different amounts of secretory products revealed by their different electron densities were frequently noticed in the apical region of granular cells.

4.4 DISCUSSION

Considerable inconsistencies have arisen over the classification of different cell types in the various salivary gland acini owing to the different techniques and criteria used for naming the cells (Review of Literature). Therefore, it was very difficult to accurately match present observations with those of other workers. However, an attempt has been made to classify the different cell types within the nomenclature used by Coons and Roshdy (1973), Binnington (1978) and Walker *et al.* (1984).

The salivary glands of *H. a. anatolicum* were composed of acini types I, II and III in females and an additional type IV in males. This agreed with the general organisation of the other ixodid ticks

(Till, 1961; Chinery, 1965; Balashov, 1972; Coons and Roshdy, 1973; Binnington, 1978; Krolak et al., 1982; Walker et al., 1984). The ultrastructure of non granular type I acini was similar in both sexes, and to that of A. americanum (Krolak et al., 1982) and R. appendiculatus (Walker et al., 1984). The complex network of basal infoldings with numerous mitochondria (Figures 4.5, 4.6 and 4.19) was typical of fluid-transporting epithelia (Pease, 1956; Fawcett, 1962). That is why Balashov (1972) and Coons and Roshdy (1973) considered this acinus type to be responsible for excretion of excess fluid during feeding. However, the transformed acinus type III appeared to be better suited to this function (Meredith and Kaufman, 1973; Megaw and Beadle, 1979). McMullen et al. (1976) suggested that the type I acini were responsible for the secretion of hygroscopic saliva which has been found to bathe the mouthparts of ixodid ticks during non parasitic stages to absorb water from an unsaturated atmosphere (Rudolph and Knülle, 1974). In addition, Rudolph and Knülle (1978) demonstrated that H. a. excavatum ticks whose acinus types II and III were almost destroyed by heavy infections with T. annulata were still able to absorb water vapours, thus further attributing this function to the type I acini.

Type I acini did not exhibit any appreciable morphological changes during feeding. However, the lipid droplets and the glycogen-like material present in unfed ticks disappeared. It was possible that they acted as energy reserves during non parasitic stages, when these acini were engaged in secreting concentrated salts to absorb water vapours from subsaturated air. The significance of numerous

Golgi bodies, associated condensing vacuoles and few electron dense granules (Figure 4.10) in the central cell was not clear.

The type II acini consisted of five granular cell types (a, b, c_1-c_3) as seen in D. variabilis (Coons and Roshdy, 1973) and R. appendiculatus (Walker et al., 1984) and in contrast to the six granular cell types of B. microplus (Binnington, 1978). However, using the electron microscope Megaw and Beadle (1979) described only two cell types in the last mentioned tick.

The a cells of type II acini and d cells of type III acini had similar ultrastructure and locations in their respective acinus types. The granules in them consisted of membrane bound sub-units with varied electron densities, bound together in a homogenous electron dense matrix. The different electron densities could possibly be due to the different developmental stages of the secretory granules. Cells similar to a, d and e cells of H. a. anatolicum have also been described in the salivary glands of D. variabilis (Coons and Roshdy, 1973), B. microplus (Binnington, 1978), A. americanum (Krolak et al., 1982) and R. appendiculatus (Walker et al., 1984). The difference found in the electron densities of sub-units might be the result of different preparation techniques, secretory properties or developmental stages. Another common feature of a and d cells was that their substructures coalesced together before secretion. Even the substructures of e cell granules were seen to be transformed at the time of excocytosis (Figure 4.44). The a, d and e cells appeared to contain the precursors of attachment cement as the timing of their secretion corresponded with the timing of cement deposition (7.3.3.1).

In addition these granules were histochemically positive for lipoproteins as was the cement cone of H. a. anatolicum (5.3.2.1). Cells analogous to a, d and e have been considered as the cement precursors in H. spinigera (Chinery, 1973) and B. microplus (Binnington, 1978).

Unlike B. microplus (Binnington, 1978) and R. appendiculatus (Walker et al., 1984), but like A. americanum (Krolak et al., 1982) there was only one d cell in acinus type III of H. a. anatolicum. This might reflect the fact that B. microplus and R. appendiculatus are ticks with short mouthparts and they have to rely on copious secretion of cement substance to ensure firm attachment as compared to H. a. anatolicum and A. americanum ticks with long mouthparts.

The sustained granular activity in the a, d and e cell, during feeding in males reflects their different physiological functions. Male ticks remain on the host for a long time during which they attach and reattach several times. In doing so they would need large reserves of precursors.

The enormous increase in the size of the type II acini during feeding was due chiefly to the enlargement of b and c cells. There were one or two b cells lying next to a cells which were easily identified by the typical capped structures of their granules. These cells corresponded with the b cells of B. microplus (Binnington, 1978) and R. appendiculatus (Walker et al., 1984) and the c_2 cells of D. variabilis (Coons and Roshdy, 1973). Next to the b cells was a group of c cell types with granules of different sizes and electron densities. Whether these differences were due to the different phases of secretory

process, different types of secretion or functionally different components is not clear. The $\underline{c}_1$ and $\underline{c}_3$ cells in female ticks remained active throughout feeding in spite of secreting most of their granules during early feeding. It is possible that they contributed to the formation of the cement cone or were perhaps essential for the establishment of initial attachment. On the other hand the b and $\underline{c}_2$ cells showed progressively increased activity with the advance of feeding. The presence of numerous secretory vacuoles alongside the Golgi bodies and abundant RER in all the b and c cells further suggested that they synthesized and secreted their products throughout feeding. All the b and c cells reacted moderately to strongly for glycoproteins and non-specific esterases, except $\underline{c}_2$ cells which did not stain for non-specific esterases (5.3.1.1-2). From their histochemical nature they appeared to be the potential candidates for anticoagulants (Ross, 1926; Kaire, 1967; Foggie, 1959; Balashov, 1972), proteolytic enzyme inhibitors (Willadsen and Riding, 1979), antihistamines (Chinery and Ayitey-Smith, 1977), prostaglandins (Dickinson et al., 1976; Higgs et al., 1976; Shemesh et al., 1979), chemoattractants for inflammatory cells (Berenberg et al., 1972), or cytolytic enzymes (Tatchell, 1971; Geczy et al., 1971).

A group of inconspicuous f cells was present at the fundus region of type III acini. At the onset of feeding these cells hypertrophied and showed a transient granular activity for unknown function. However, they transformed markedly in their external morphology to undertake the function of excess fluid excretion by forming an extensive

basolateral labyrinth with the enlarged ablumenal interstitial cells (Figures 4.34 and 4.35) similar to B. microplus (Megaw and Beadle, 1979) and R. appendiculatus (Fawcett et al., 1981b).

The observation on interstitial cells, which separated granular cells from each other, conformed well with the observations of Meredith and Kaufman (1973), Megaw and Beadle (1979) and Fawcett et al. (1981b). The interstitial cells were present in two tiers: the adlumenal and ablumenal interstitial cells (Fawcett et al., 1981b), similar to the cap and water cells of Meredith and Kaufman (1973) and Megaw and Beadle (1979). The present study confirmed the observations of Krolak et al. (1982), that there was only one adlumenal interstitial cell in all the granular acinar types winding its way all around the acinar lumen alternating with the granular cells. These interstitial cells were inconspicuous in the unfed ticks but became more prominent during feeding. The ablumenal interstitial cells of type III acini increased enormously in size in feeding females and formed a labyrinthine system of extraordinary complexity along with transformed f cells to provide an increased surface facing the haemolymph. Similar labyrinthine structures have been described in the type III acini of feeding B. microplus (Megaw and Beadle, 1979) and R. appendiculatus (Fawcett et al., 1981b) and have been assigned the role of fluid excretion during feeding. However, in contrast to R. appendiculatus (Fawcett et al., 1981b) the ablumenal interstitial cells in H. a. anatolicum made the major contribution to the formation of the basal labyrinth.

Similar but less pronounced changes in the interstitial cells of type II acini of females and all the granular acini of males might

explain their different functions. They could be involved in excretion of excess fluid to a small extent but it is more likely that they function to wash the granular products down to the salivary duct.

The gap junctions between the adlumenal and ablumenal interstitial cells might play an important role in fluid transport. Similar junctions might also be present between the f cells and the ablumenal interstitial cells (Fawcett et al., 1981b) but were not observed in this study.

The ultrastructure of the g cells of type IV acini was similar to that of B. microplus (Binnington, 1978) and R. appendiculatus (Walker et al., 1984). The secretions of these cells have been postulated to be involved in the smooth transfer of the spermatophore during mating (Feldman-Muhsam et al., 1970).

This study describes for the first time the process of granule secretion in the salivary glands of ixodid ticks. Unlike the soft tick Argas arboreus (Coons and Roshdy, 1981) the granular acini of H. a. anatolicum discharge their granular contents exclusively by exocytosis. The transformation of granular sub-units to an amorphous state suggested that there is at least partial solubilization of the granular contents before discharge. How this solubilization occurs is not clear. This along with the evidence of discharge through intracellular channels connecting the secretory granules (Figures 4.46 and 4.47) explained how the big granules of a, d, e and g cells could be secreted without being physically moved to the apical end. Mechanisms, analogous to this have been reported for mast-cell degranulation (Rohlich et al., 1971).

4.5 SUMMARY

The histology and ultrastructure of the salivary glands of male and female H. a. anatolicum ticks have been examined in unfed and feeding ticks with special emphasis on aspects related to the feeding process. The salivary glands of H. a. anatolicum consisted of three types of acini (acinus I, II and III) in females and an additional type IV acinus in males. The type I acinus was agranular and did not show any apparent morphologic changes during feeding. The presence of a basal labyrinth having a number of closely associated mitochondria supported the hypothesis that these acini secrete hygroscopic saliva during questing stages to absorb water from an unsaturated atmosphere. There were five granular cell types (a, b, c_1-c_3) in type II acinus, three granular cells types (d, e and f) in type III acinus, and one type granular cell (g) in type IV acinus. The cells a, d and e secreted most of their granules during early feeding and were considered as cement precursors. The b and c cells appeared to synthesize and secreted their products throughout feeding and were considered to secrete anticoagulants, enzymes and other inflammatory agents required during feeding. The type III acinus in females showed remarkable cell transformations, during the course of feeding. There was an enormous increase in the secretory granules of g cells as the feeding advanced. The secretory granules were released by a process of exocytosis, by direct fusion with the apical membranes and through channels interconnecting several granules. The interstitial cells which were insignificant in acinar types II, III and IV of unfed ticks became more distinct during feeding. The

ablumenal interstitial cells of type III acinus, in females showed an enormous increase in size during feeding. They formed a basal labyrinth of extraordinary complexity by interdigitating with the basolateral membranes of transformed f cells to form a network of extracellular channels to excrete excess fluid during feeding, as has been suggested for other ixodid ticks.

Figure 4.1 Acini types I, II and III of an unfed female. Methacrylate section (Giemsa, x 272).

Figure 4.2 Type II acini, female, 72 hours after attachment. Methacrylate section (Giemsa, x 544).

I = type I acinus; II = type II acinus;

III = type III acinus; a = a cell;

b = b cell; c = c cells.

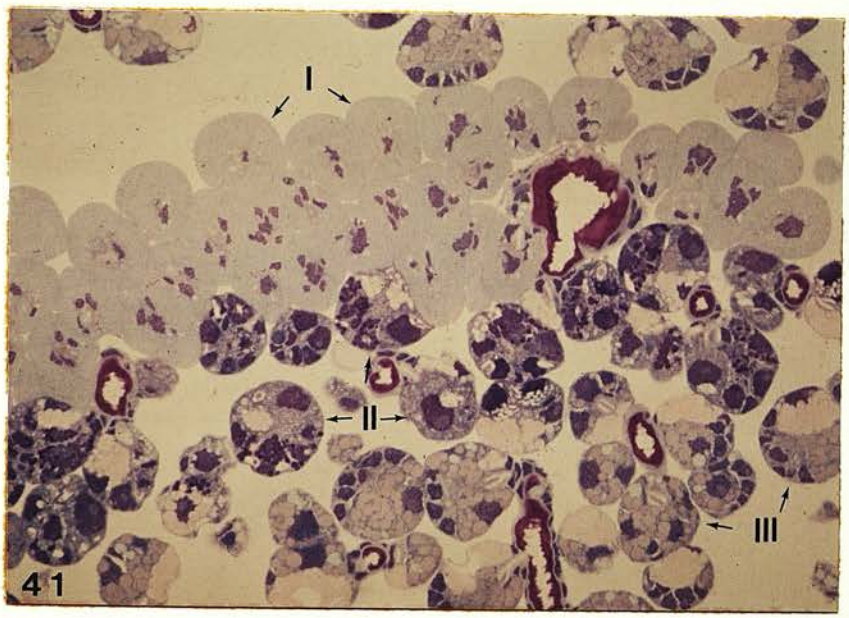

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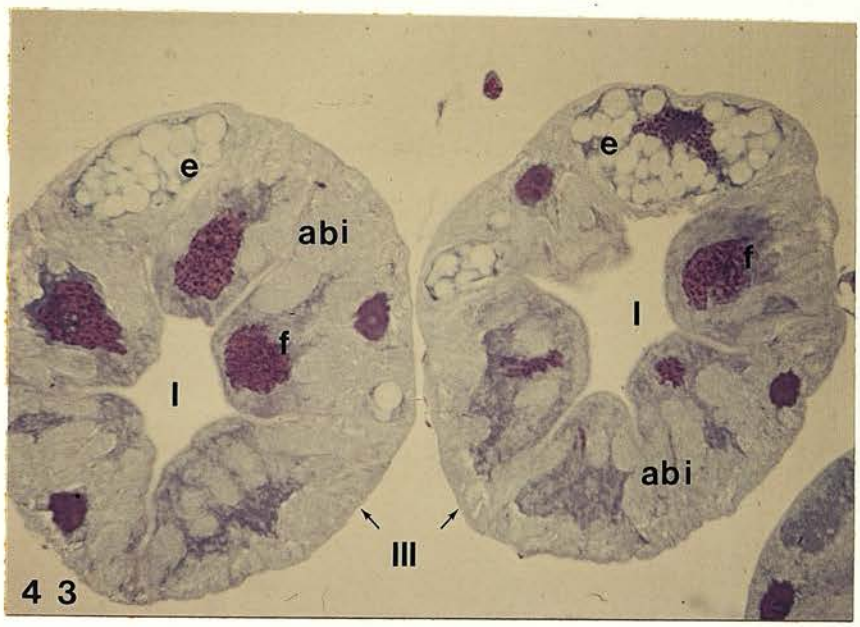
Figure 4.3 Type III acini, female, 144 hours after attachment. Note the well developed lumen with brush border. Methacrylate section (Giemsa, x 544).

Figure 4.4 Acini types III and IV, male, 144 hours after attachment. Methacrylate section (Giemsa, x 544).

III = acinus type III; IV = acinus type IV;

e = e cells; f = f cells; g = g cells;

abi = ablumenal interstitial cell; l = lumen.



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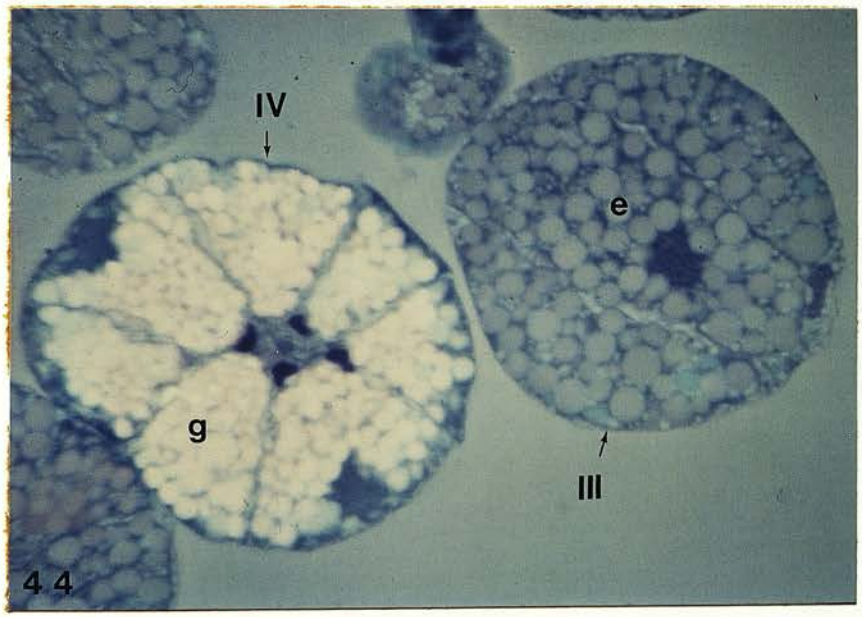

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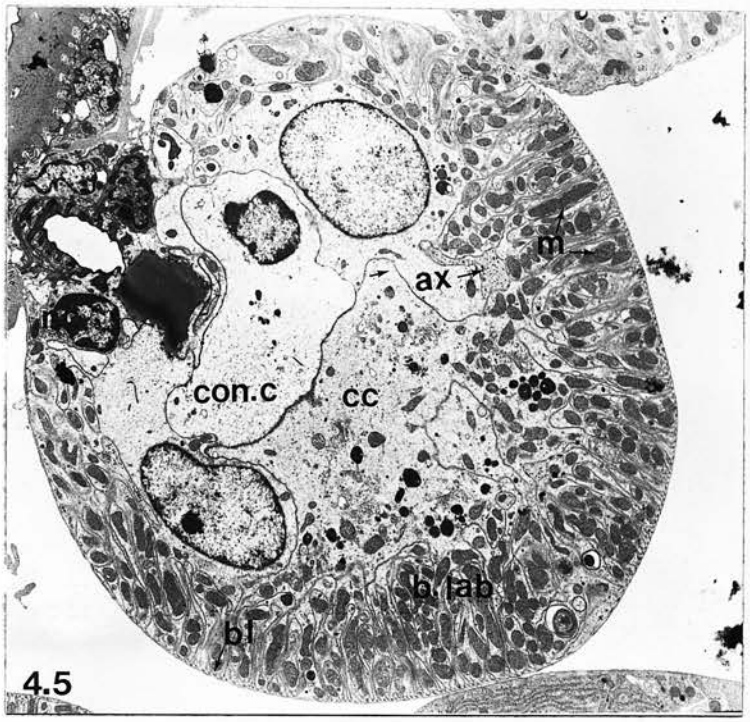
Figure 4.5 Type I acinus, female, 72 hours after attachment. (EM, x 3,775)

Figure 4.6 Type I acinus, male, 144 hours after attachment. (EM, x 10,780)

Figure 4.7 Type I acinus, female, 72 hours after attachment. (EM, x 10,780)

ad = acinar duct; ax = axon with neurosecretory vesicles; bl = basal lamina; b.lab. = basal labyrinth; cc = central cell; con.c = constrictor cell; edb = electron dense bodies; fs = filamentous structures; gb = Golgi body; m = mitochondria; mb = myelin bodies; mt = microtubular mat; nc = neck cell; pc = peripheral cells.



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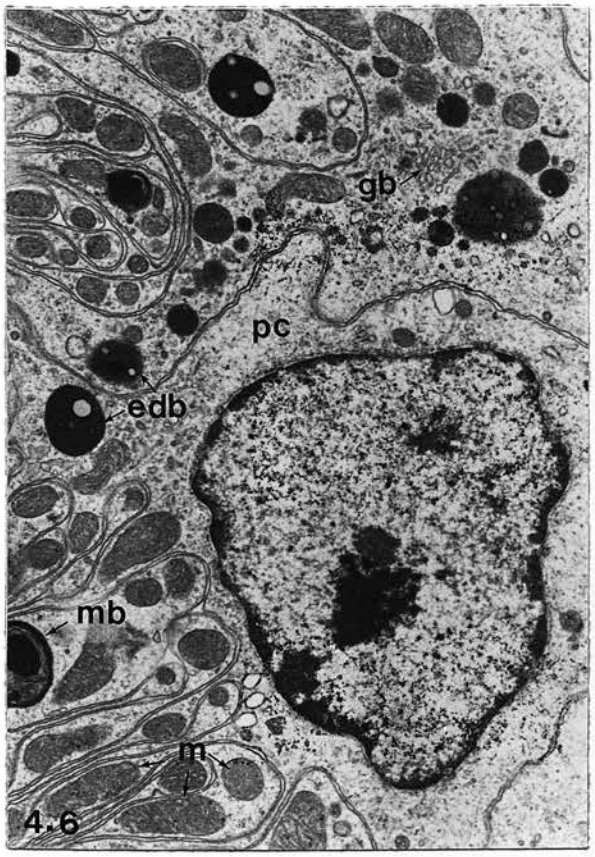

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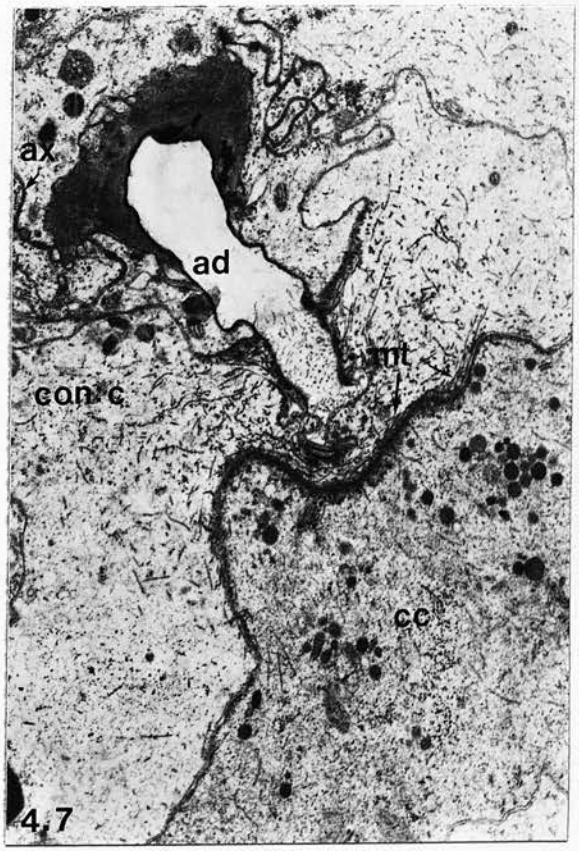

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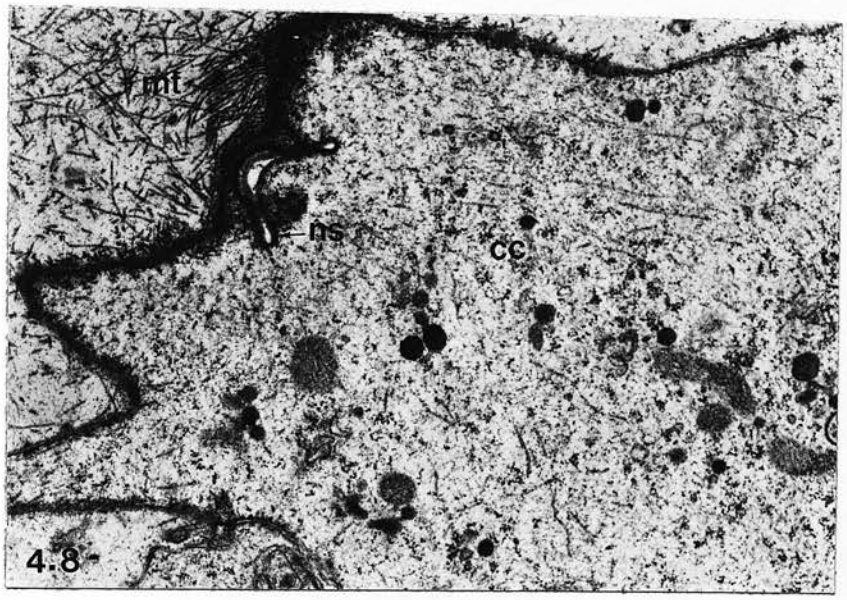
Figure 4.8 Type I acinus, female, 144 hours after attachment. (EM, x 14,000)

Figure 4.9 Type I acinus, unfed female. (EM, x 6,440)

Figure 4.10 Type I acinus, female, 144 hours after attachment. (EM, x 17,500)

bl = basal lamina; b.lab. = basal labyrinth; cc = central cell; con.c = constrictor cell; edb = electron dense bodies; gb = Golgi body; ld = lipid droplet; m = mitochondria; mt = microtubular mat; ns = nipple structure; pc = peripheral cell.



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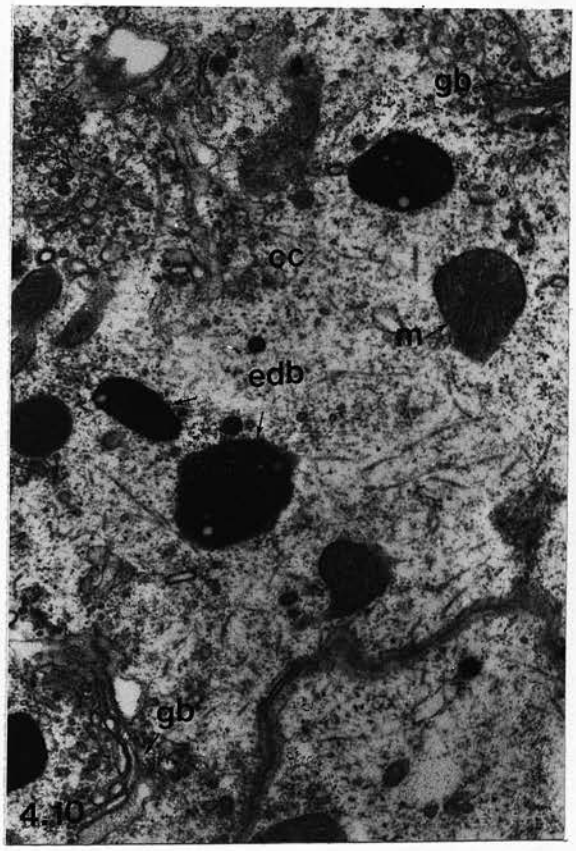

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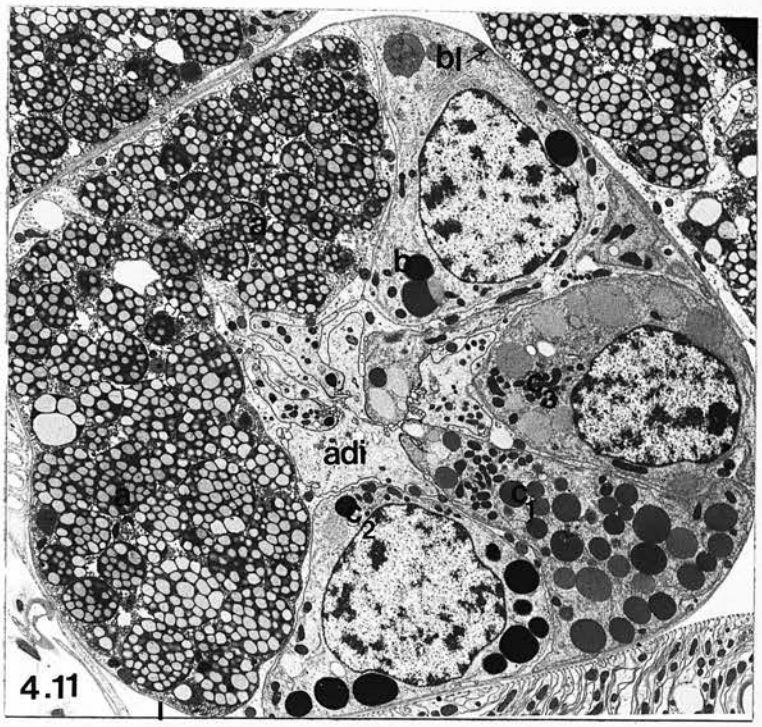
Figure 4.11 Type II acinus, unfed female. (EM, x 3,575)

Figure 4.12 Type II acinus, female, 72 hours after attachment. (EM, x 2145)

a = a cell; abi = ablumenal interstitial cell; adi = adlumenal interstitial cell; b = b cell; bl = basal lamina; c_1 = c_1 cells; c_2 = c_2 cell; c_3 = c_3 cell; l = lumen.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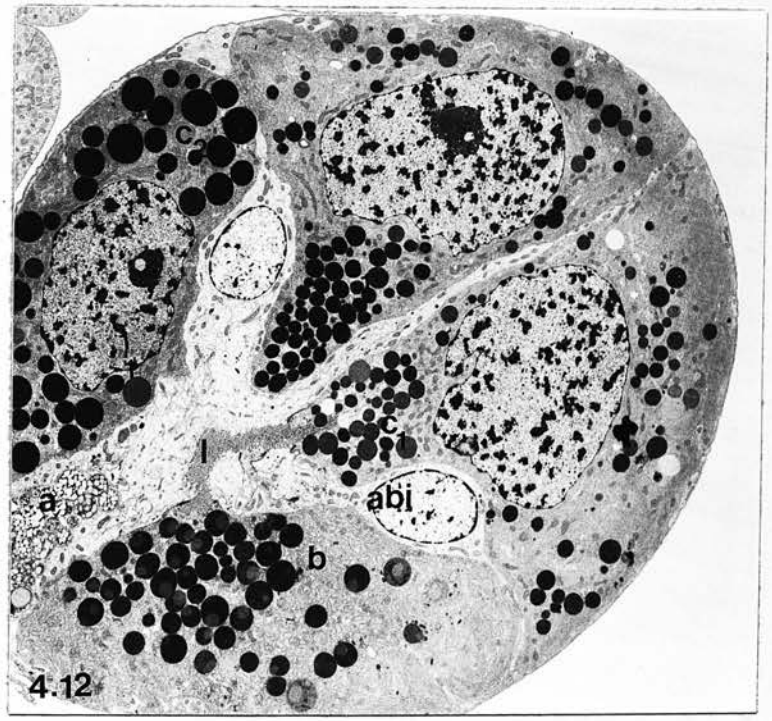
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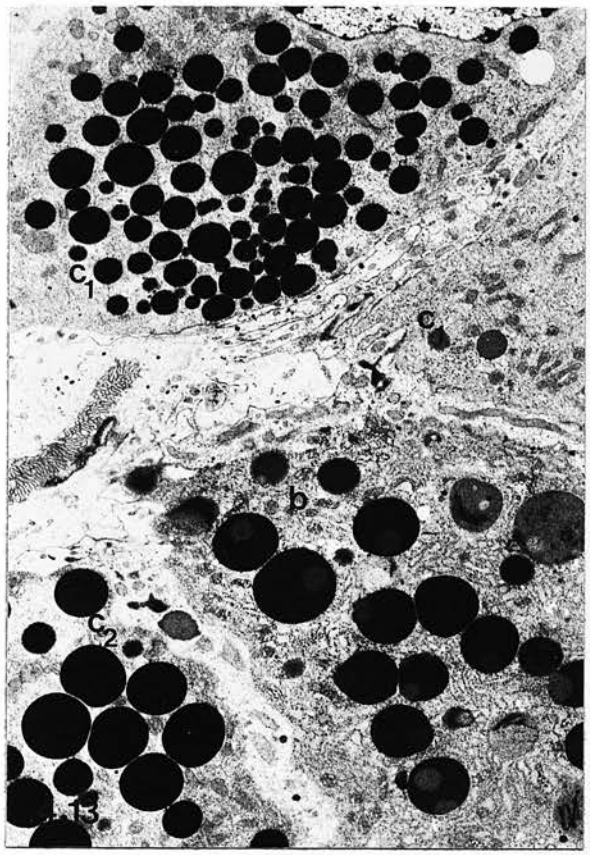
Figure 4.13 Type II acinus, female, 72 hours after attachment. (EM, x 3,850)

Figure 4.14 Type II acinus, male, 72 hours after attachment. (EM, x 6,440)

Figure 4.15 a cell granules, unfed female. Note membrane-like outlines around the granule sub-units (arrows). (EM, x 23,100)

Figure 4.16 Type b cell, unfed female. (EM, x 14,000)

a = a cell; adi = adlumenal interstitial cell; b = b cell; c_1 = c_1 cell; c_2 = c_2 cell; c_3 = c_3 cell; fs = filamentous structures; m = mitochondria; n = nucleus; rer = rough endoplasmic reticulum; sg = secretory granule.



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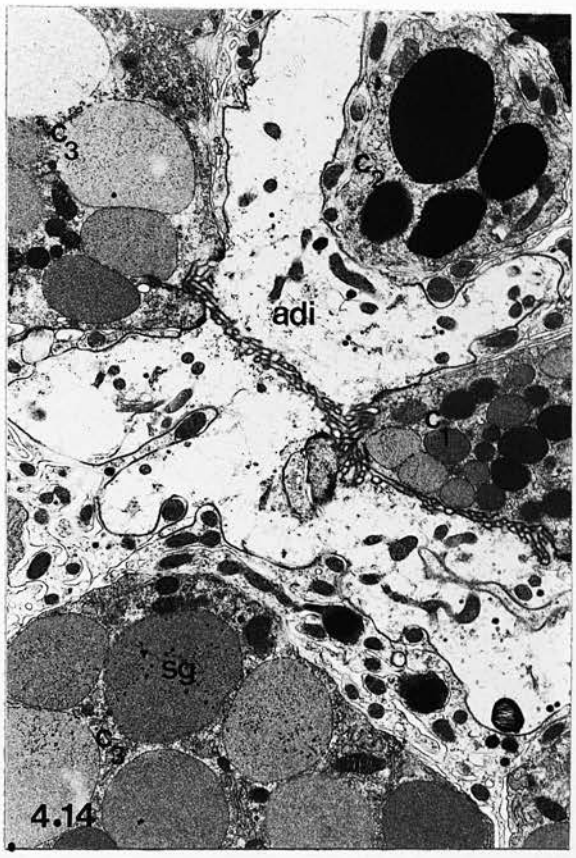

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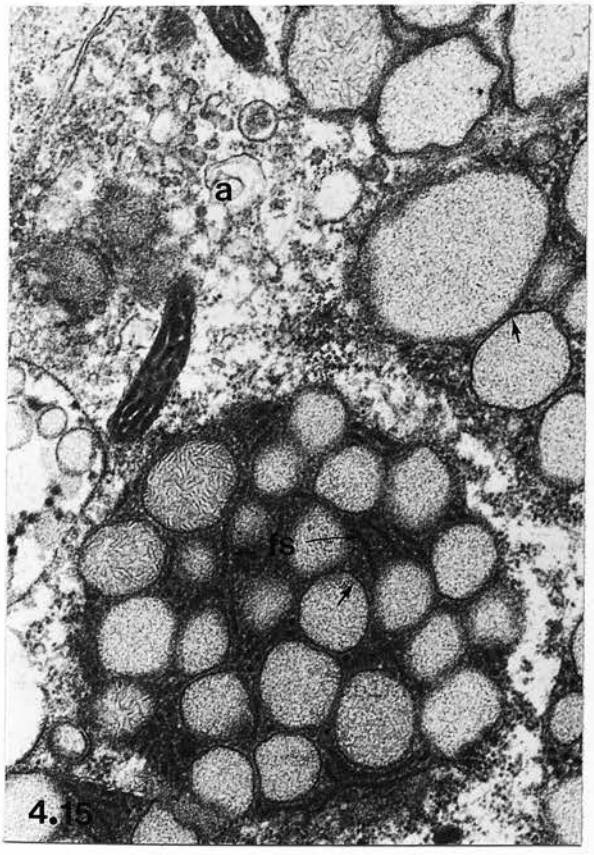

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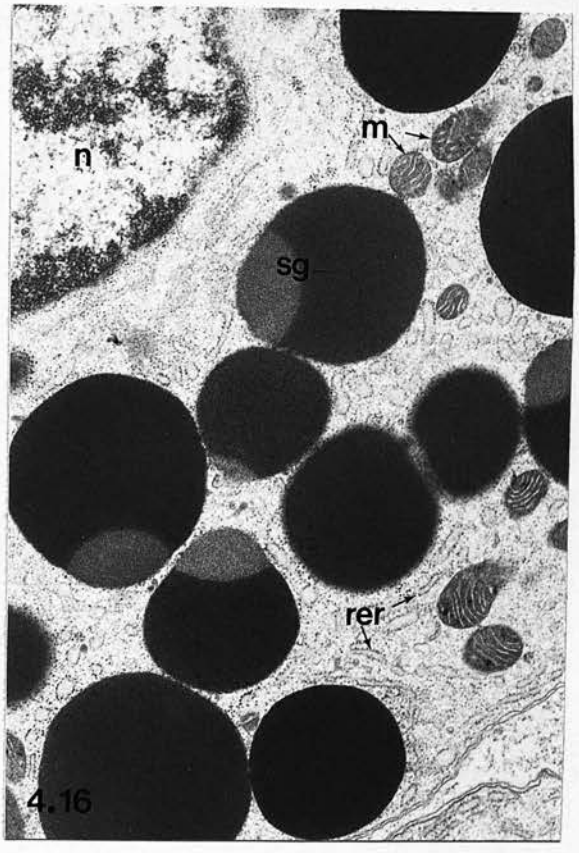


4.16

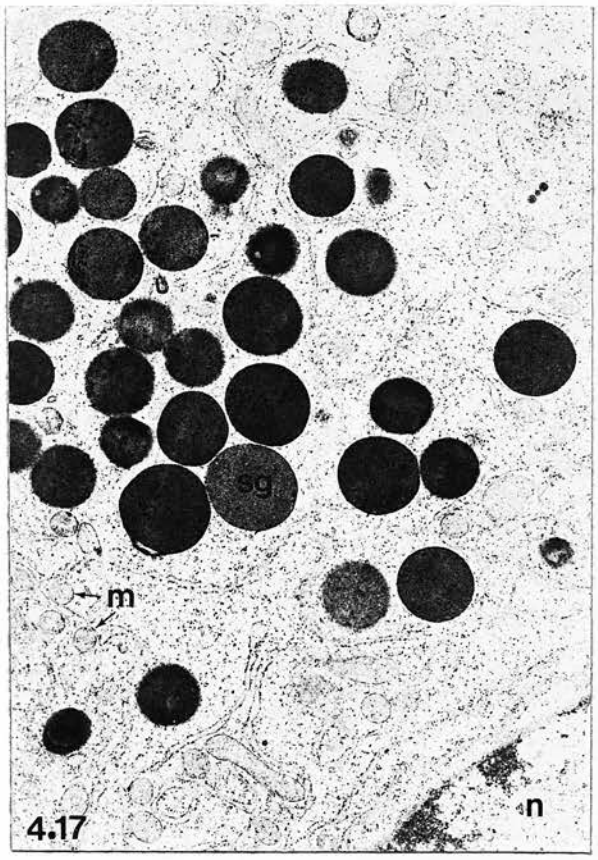
Figure 4.17 Type c_1 cell, female, 72 hours after attachment. (EM, x 8,400)

Figure 4.18 Type c_1 cell, unfed female. (EM, x 14,000)

Figure 4.19 Type c_1 cell, unfed male. (EM, x 17,500)

Figure 4.20 Type c_1 cell, male, 144 hours after attachment. (EM, x 10,780)

gb = Golgi body; m = mitochondria; n = nucleus; rer = rough endoplasmic reticulum; sg = secretory granules.



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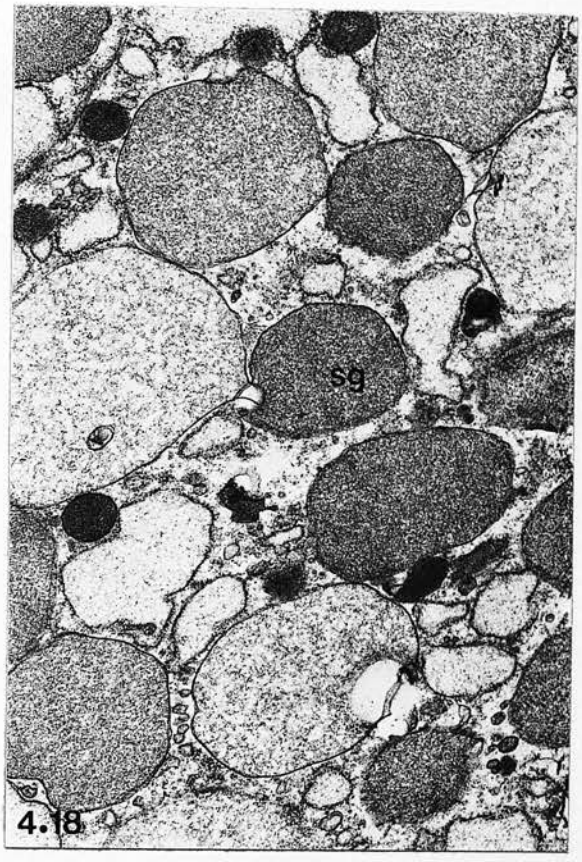

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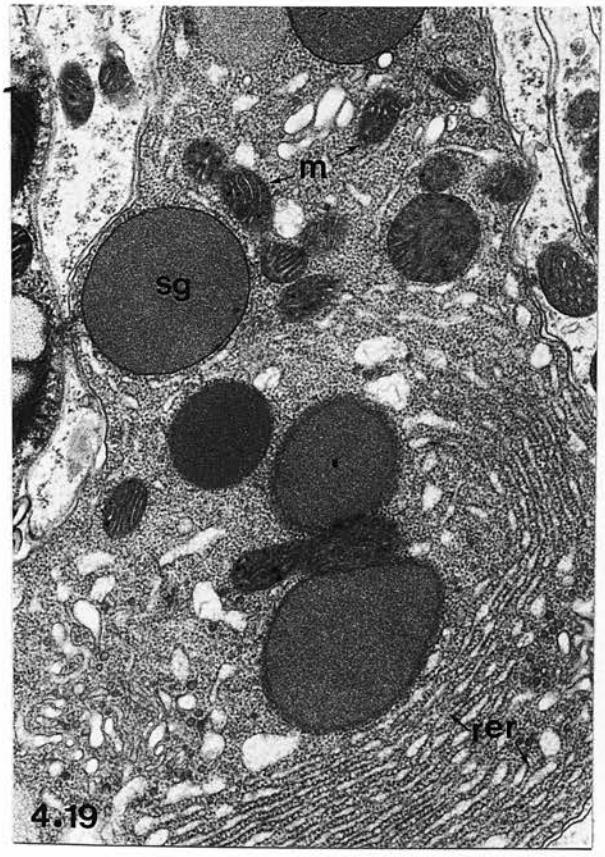

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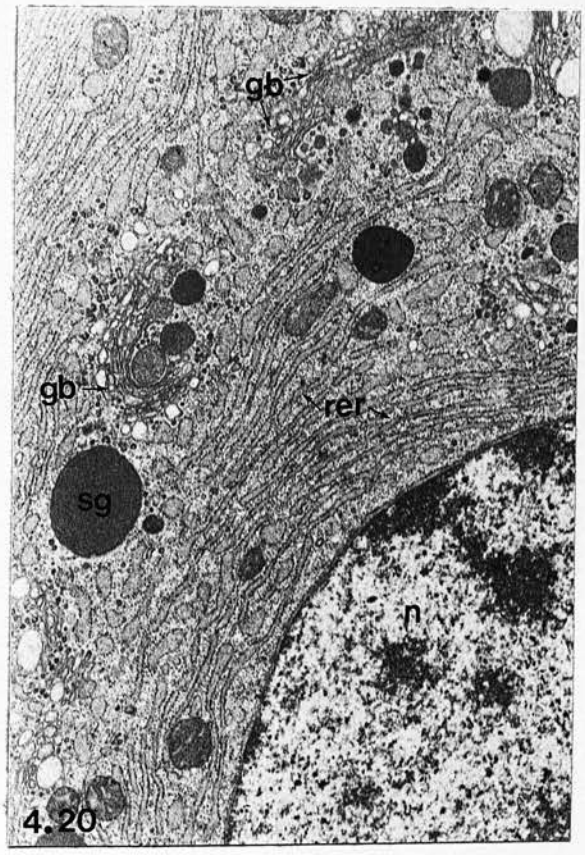


4.20

Figure 4.21 As Figure 4.20, high power view of the Golgi body. (EM, x 38,500)

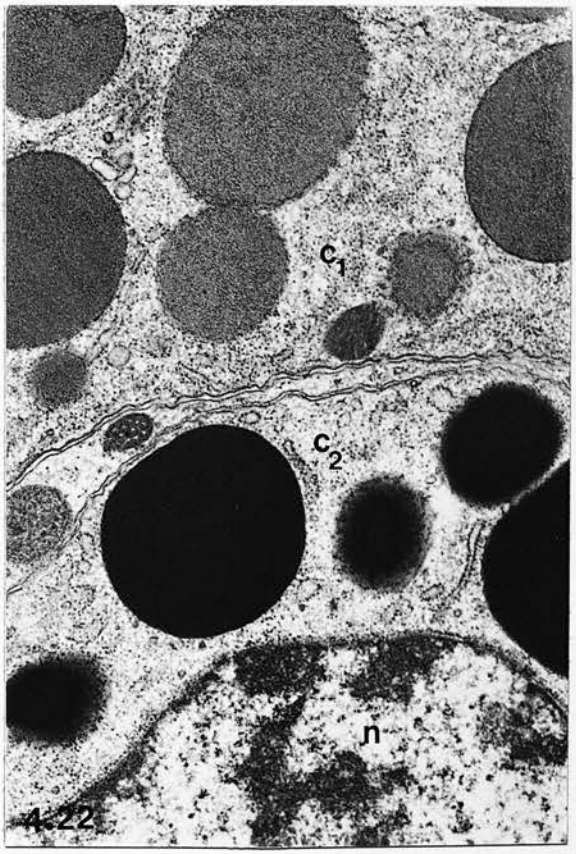
Figure 4.22 Type $\underline{c}_1$ and $\underline{c}_2$ cells, unfed female. (EM, x 17,500)

Figure 4.23 Type II acinus, female, 72 hours after attachment. (EM, x 23,100)

Figure 4.24 Type $\underline{c}_2$ cell, male, 72 hours after attachment. (EM, x 17,500)

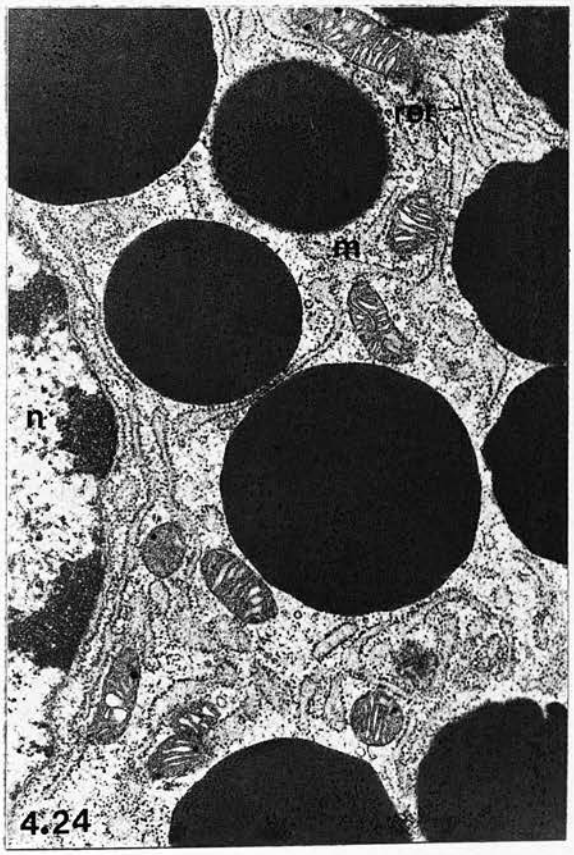
adi = adlumenal interstitial cell; gb = Golgi body; l = lumen; m = mitochondria; n = nucleus; rer = rough endoplasmic reticulum; sd = septate desmosome junction; sg = secretory granules.

4.21



4.22

4

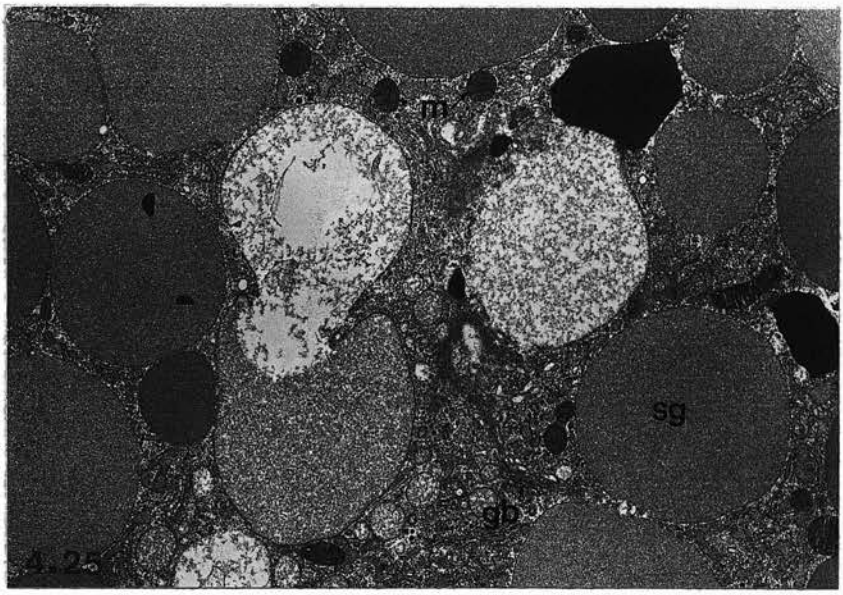


4.24

Figure 4.25 Type c_3 cell, female, 72 hours after attachment. (EM, x 8,400)

Figure 4.26 As Figure 4.25, high power view. (EM, x 23,100).

gb = Golgi body; m = mitochondria; rer = rough endoplasmic reticulum; sg = secretory granules.


m
sg
gb
4.25

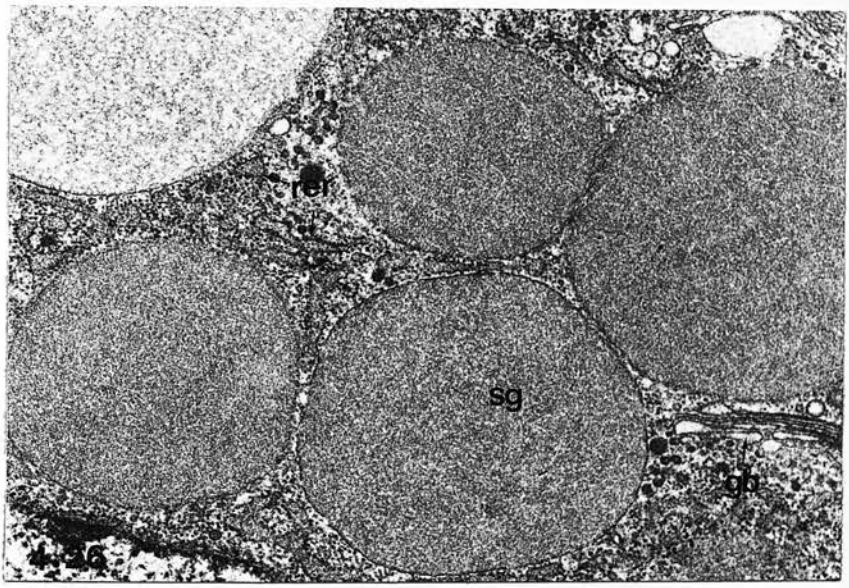
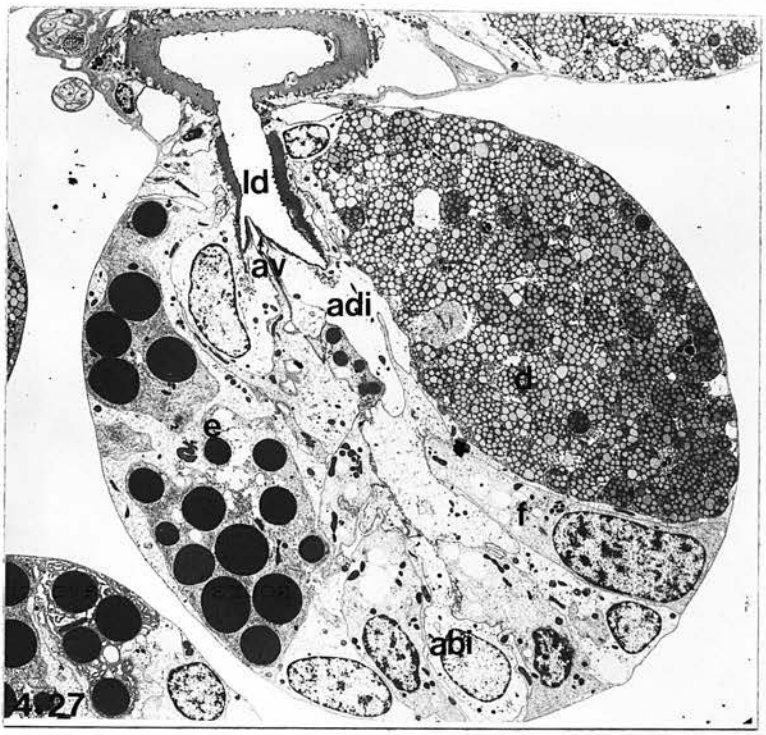

rer
sg
gb
4.26

Figure 4.27 Type III acinus, unfed female. (EM, x 2,145)

Figure 4.28 Type e cell, female, 72 hours after attachment. (EM, x 6,440)

Figure 4.29 As Figure 4.28, high power view of e cell secretory granule. (EM, x 23,100)

abi = ablumenal interstitial cell; adi = adlumenal interstitial cell; av = acinar valve; d = d cell; e = e cell; f = f cell; ld = lobular duct; m = mitochondria; rer = rough endoplasmic reticulum; sg = secretory granules.



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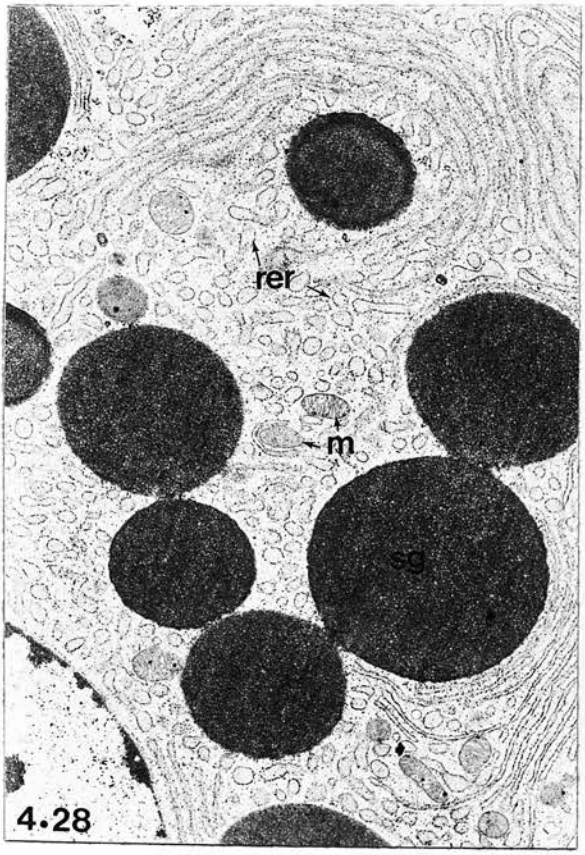

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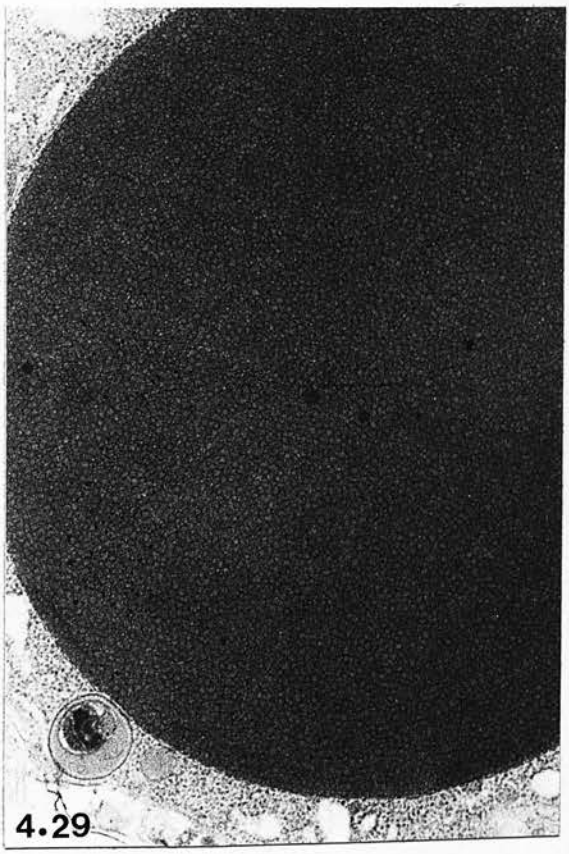

4.29

Figure 4.30 Type f cell, female, 72 hours after attachment. (EM, x 8,400)

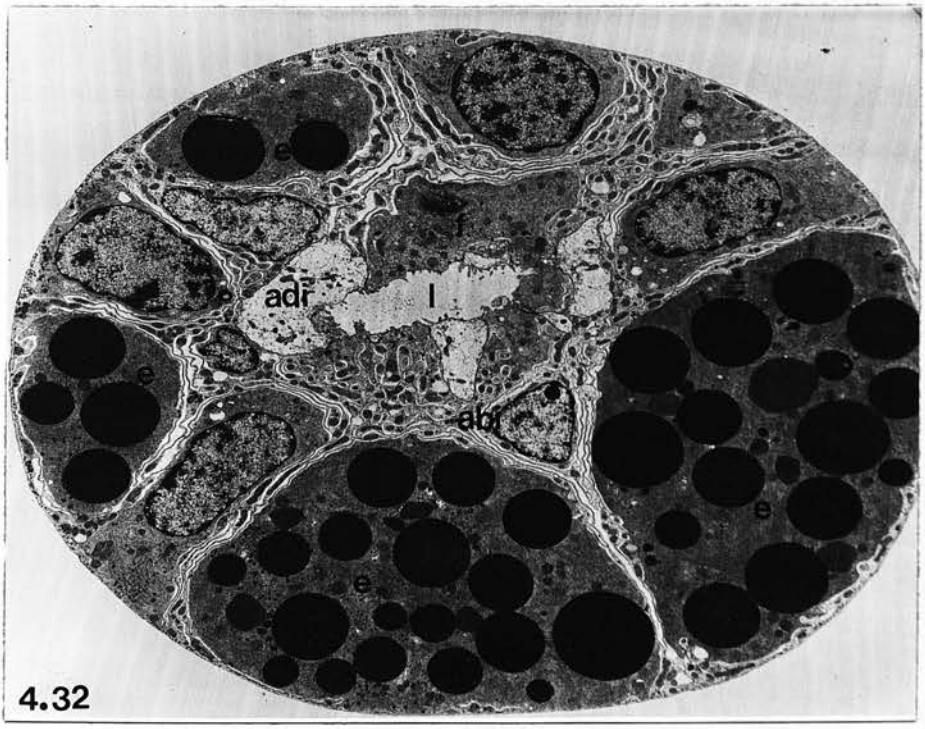
Figure 4.31 Type III acinus, male, 144 hours after attachment. (EM, x 6,440)

Figure 4.32 Type III acinus, male, 144 hours after attachment. (EM, x 2,310)

abi = ablumenal interstitial cell; adi = adlumenal interstitial cell; e = e cells; f = f cells; gb = Golgi body; l = lumen; m = mitochondria; rer = rough endoplasmic reticulum; sg = secretory granules.

4.30

4.31



4.32

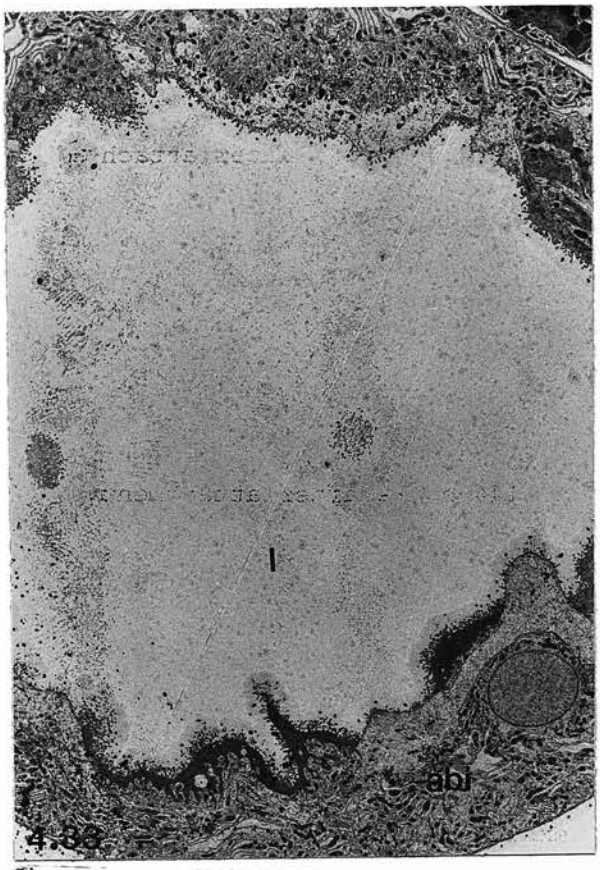
Figure 4.33 Type III acinus, female, 144 hours after attachment. (EM, x 1,001)

Figure 4.34 Type f cell, female, 144 hours after attachment. (EM, x 4,900)

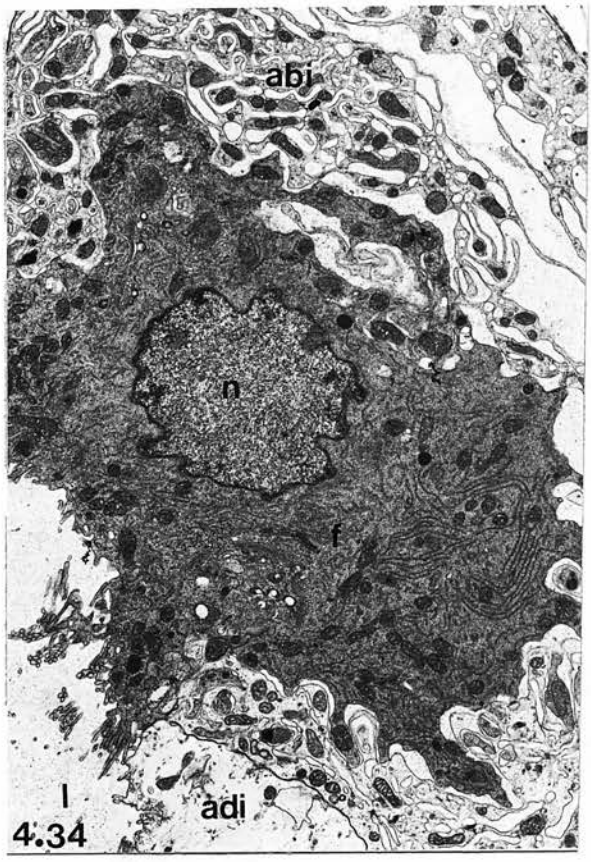
Figure 4.35 Type III acinus, female, 144 hours after attachment. (EM, x 2,310)

Figure 4.36 Type III acinus, interface between the adlumenal interstitial cell and ablumenal interstitial cell. (EM, x 49,700)

abi = ablumenal interstitial cell; adi = adlumenal interstitial cell; bl = basal lamina; f = f cells; gp = gap junction; l = lumen; m = mitochondria; n = nucleus.



4.33



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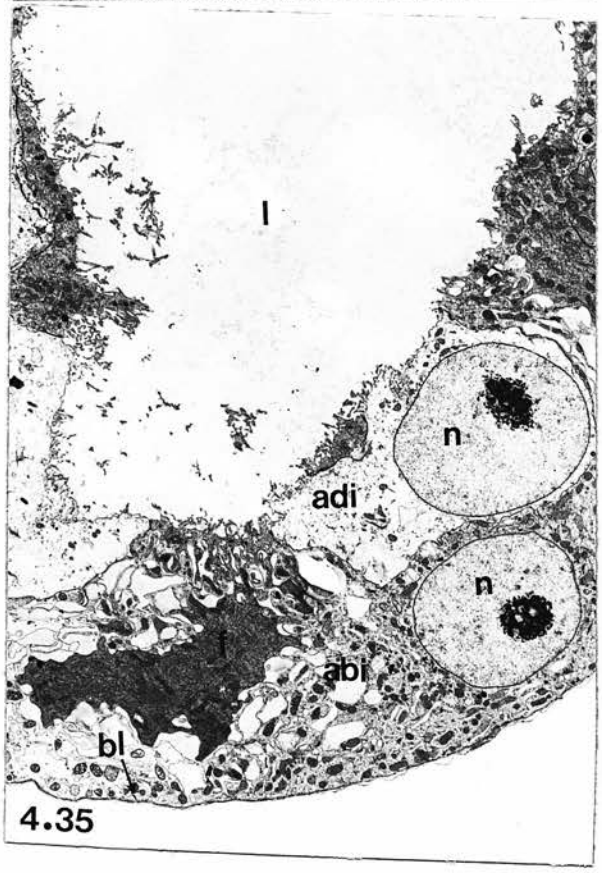

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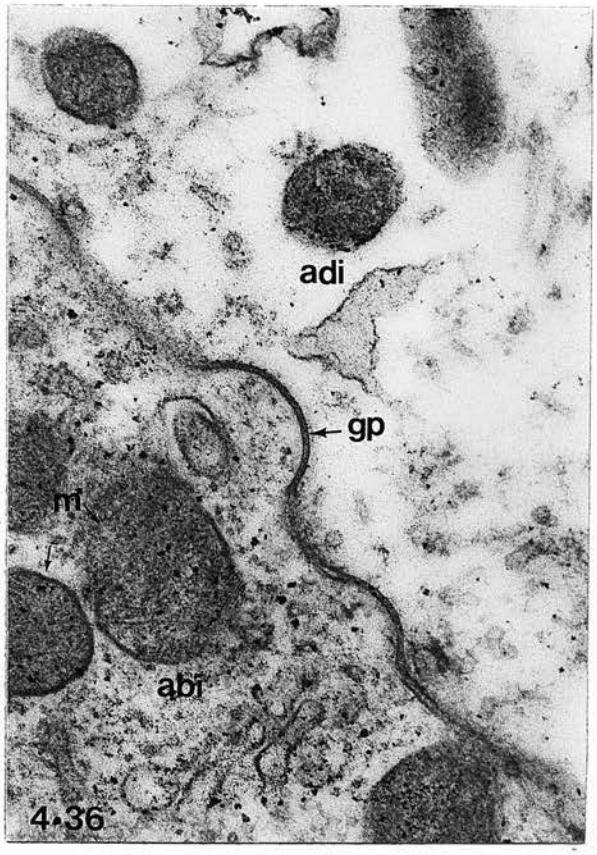
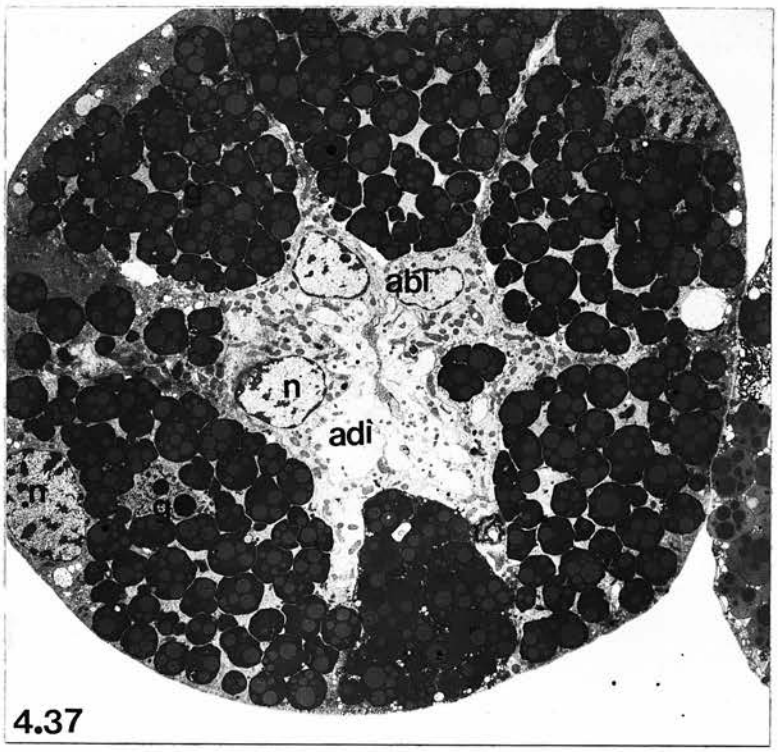


4.36

Figure 4.37 Type IV acinus, male, 144 hours after attachment. (EM, x 2,145)

Figure 4.38 Type g cell, male, 144 hours after attachment. (EM, x 6,440)

abi = ablumenal interstitial cell; adi = adlumenal interstitial cell; g = g cells; n = nuclear; sg = secretory granules.



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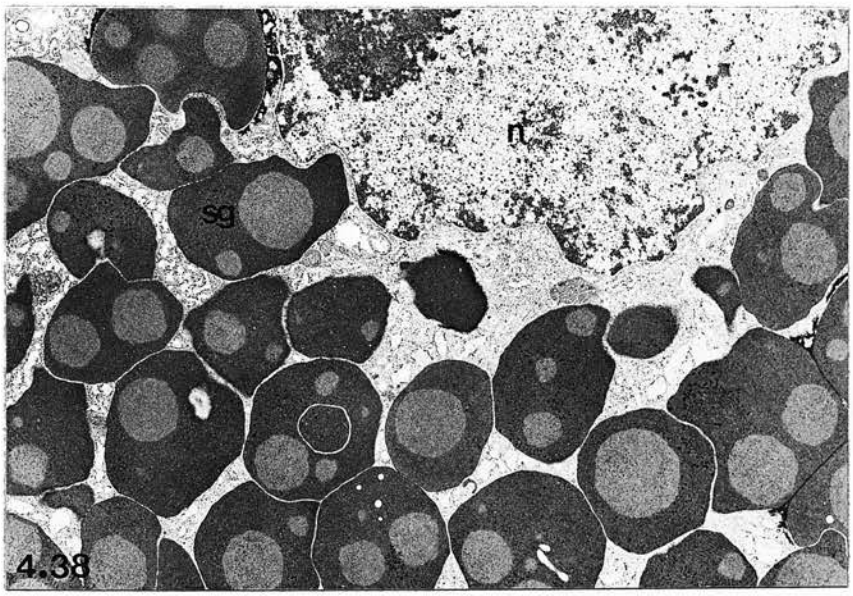


4.38

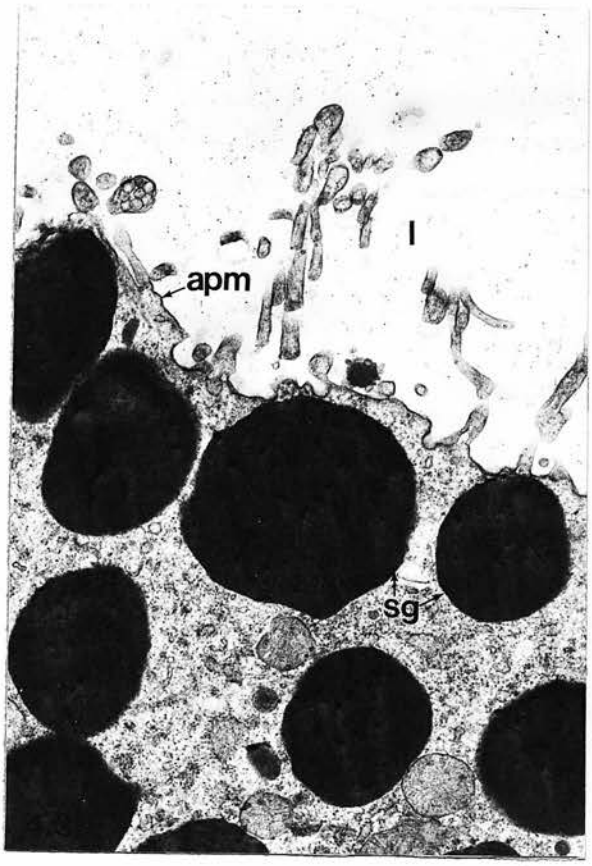
Figure 4.39 Type c cell, female, 72 hours after attachment. Note numerous secretory granules aligned along the apical plasma membrane of the cell. (EM, x 17,500)

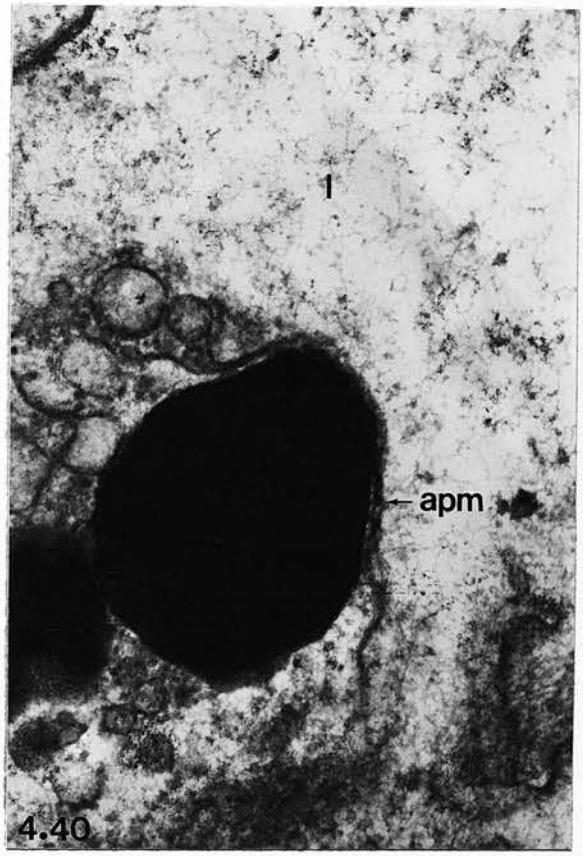
Figure 4.40 Type c cell, female, 144 hours after attachment. Note a secretory granule in intimate apposition to the apical plasma membrane. (EM, x 49,700)

Figure 4.41 Type c cell, female, 144 hours after attachment. Note the secretory granule at arrows. (EM, x 17,500)

Figure 4.42 As Figure 4.41, high power view. (EM, x 64,400).

apm = apical plasma membrane; l = lumen; mv = microvilli; sg = secretory granules.





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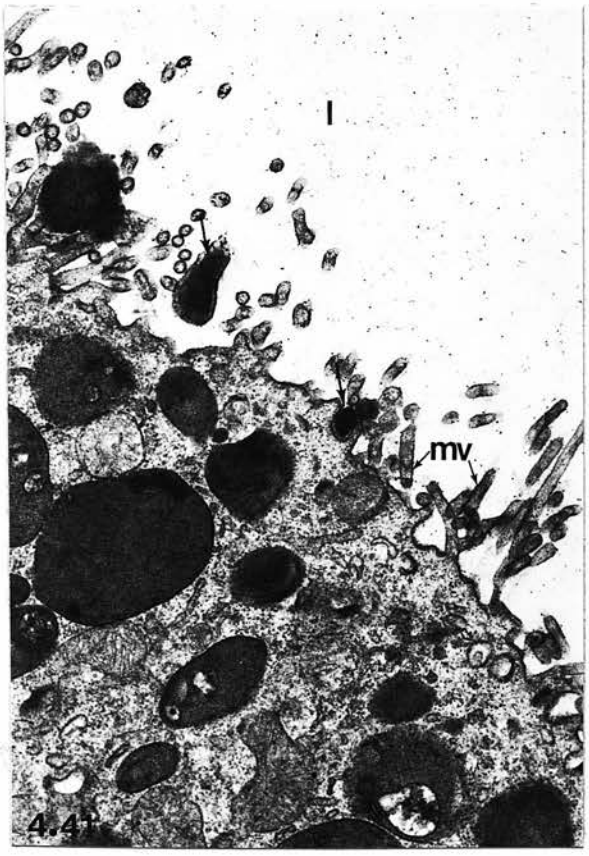


4.41

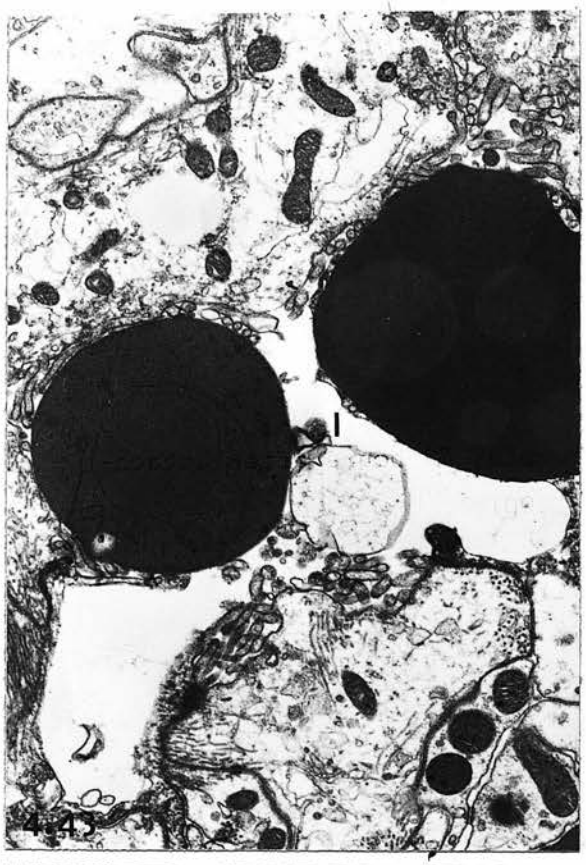
Figure 4.43 Type IV acinus, male, 144 hours after attachment. (EM, x 14,000)

Figure 4.44 Type III acinus, male, 72 hours after attachment. (EM, x 30,100)

Figure 4.45 Type III acinus, female, 24 hours after attachment. (EM, x 23,100)

Figure 4.46 Acinus II, female, 144 hours after attachment. Note many secretory granules along the lumenal border of c cells appear partly to completely empty. (EM, x 4,970).

asg = a cell secretory granules; csg = c cell secretory granules; esg = e cell secretory granules; l = lumen.



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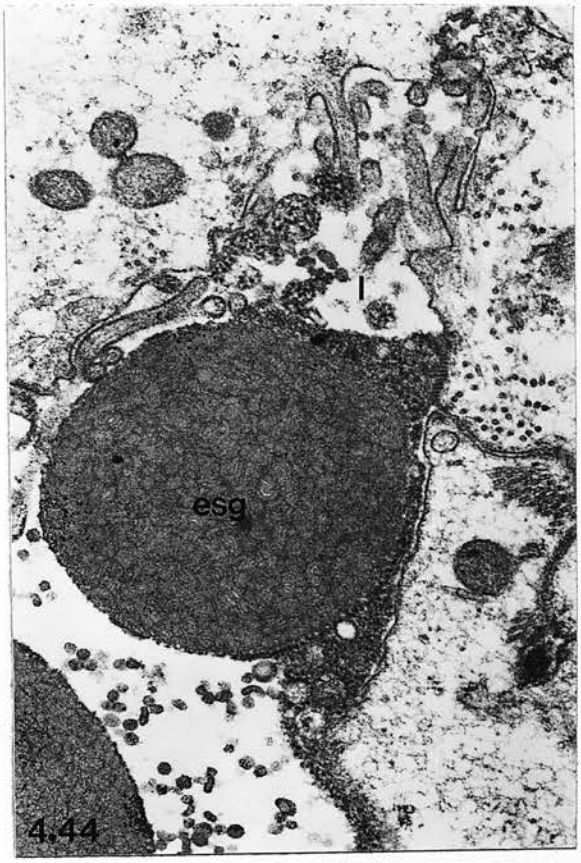

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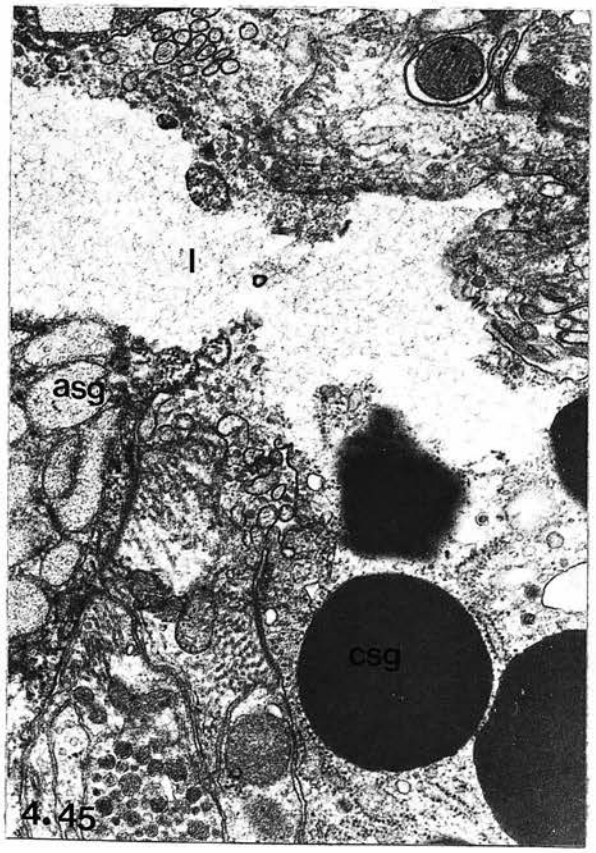
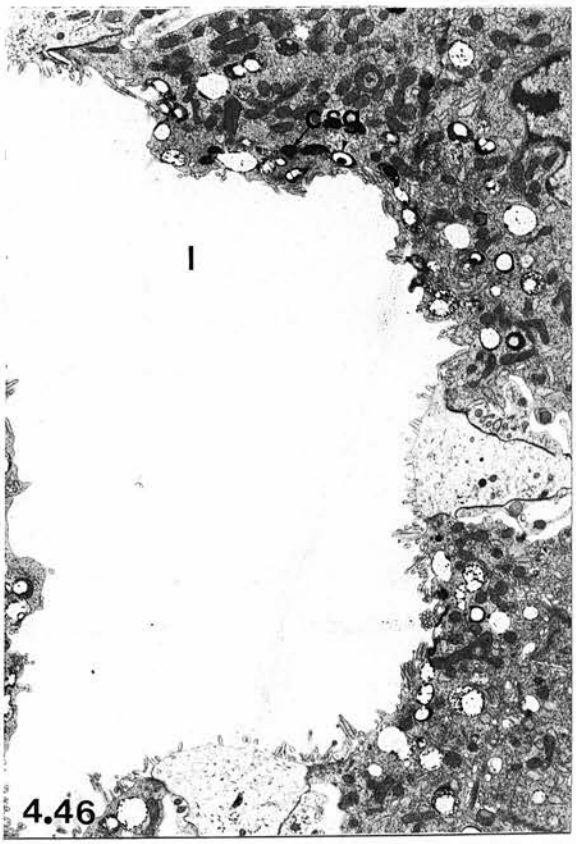


4.45



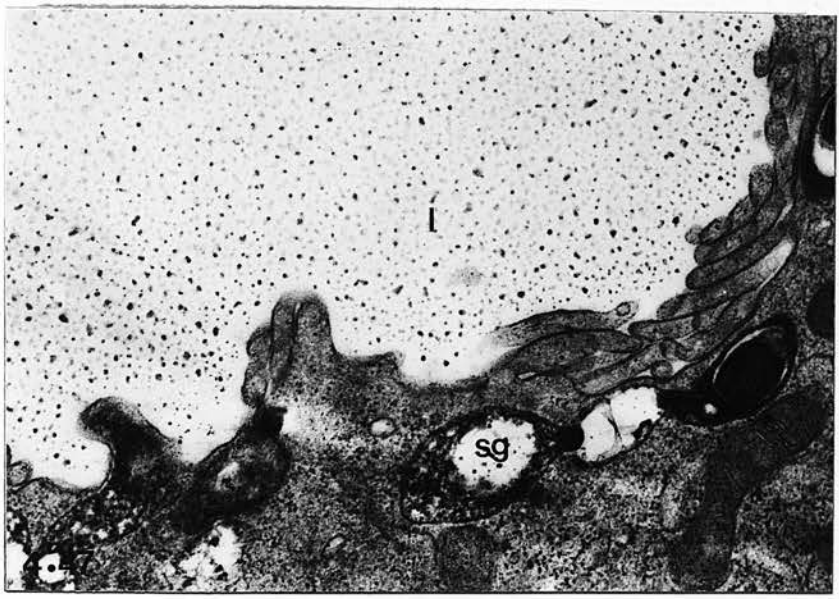
4.46

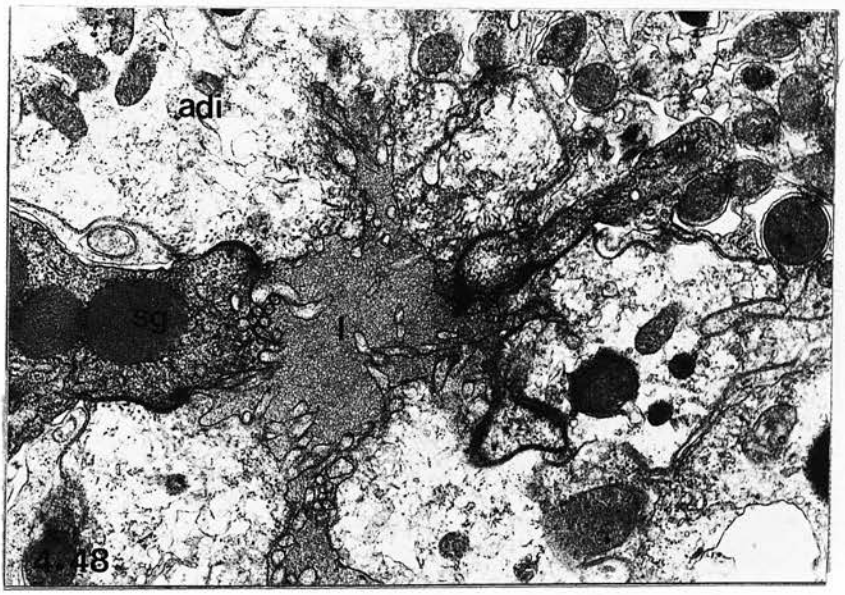
Figure 4.47 As Figure 4.46, high power view.
(EM, x 23,100)

Figure 4.48 Type II acinus, female, 72 hours after attachment showing the acinar lumen filled with secretory material
(EM, x 14,000).

adi = adlumenal interstitial cells;

l = lumen; sg = secretory granules.


l
sg


adi
sg
l

CHAPTER FIVE

HISTOCHEMICAL STUDIES ON THE SALIVARY GLANDS AND TICK FEEDING SITES

CONTENTS

5.1 INTRODUCTION

Despite extensive studies on the salivary glands of ixodid ticks the specific nature of their potential secretory products and their role in feeding are relatively unstudied. Binnington (1978) and Walker et al. (1984) have provided the most comprehensive accounts regarding the nature of the salivary components and suggested specific functions for some of the presumed secretory products. However, in only a few instances have particular components been shown to occur in the tick saliva or at tick feeding sites and in no situation is it certain if these components played any definitive role in the development of the feeding lesion or in the process of tick feeding.

The present study aimed to identify the potential secretory products in the salivary glands of H. a. anatolicum and to locate them at tick feeding sites if they were secreted during feeding. It is presumed that these components, if secreted into host tissues, might also be important antigens. The anticipated information would facilitate understanding of the immune response of the host and its relationship to mechanisms determining the degree of resistance to tick infestation.

5.2 EXPERIMENTAL DESIGN

To study the distribution pattern of the proteins, polysaccharides, lipids and nucleic acids in the salivary glands, two types of preparations were employed: whole salivary glands, and methacrylate-embedded, 1-2μ thick sections. For enzyme histochemistry, pre-incubated methacrylate-embedded sections were also included. For

each test salivary glands from at least three ticks of each sex, each from unfed, 24, 72 and 144 hours fed ticks were examined. The histochemical tests and controls used are summarised in Tables 1 and 2. The histochemical methods employed were based on those of Bancroft (1975), Pearse (1972) and Chayen et al. (1969) with some modifications (see Materials and Methods).

For histochemistry of tick feeding sites two types of preparations were used: cryostat sections and methacrylate-embedded sections (see Materials and Methods). Sections from at least 20 tick feeding sites, each at 24, 72 and 144 hours post attachment, were examined for each tick. Histochemical methods employed were similar to those used on salivary glands.

The reactions obtained following histochemical tests were graded as follows: very strong (4), strong (3), moderate (2), weak (1), faint (±) and negative (0). The results obtained from these two studied were compared.

5.3 RESULTS

5.3.1 Histochemistry of the Salivary Glands

5.3.1.1 Proteins, lipids and polysaccharides: The agranular cytoplasm of type I acini gave a moderate, diffuse to speckled reaction for basic proteins, lipids and polysaccharides.
The intensity of staining for polysaccharides and lipids was considerably reduced as the feeding progressed (Table 5.3). Treatment with diastase had little effect on polysaccharide staining. In addition the cytoplasm stained weakly for tryptophan, sulphydryl groups and ribonucleic acid.

Table 5.1 Histochemical methods used on the salivary glands of male and female H. a. anatolicum

Substance demonstrated	Method	Process		Positive controls	Negative controls
		Sections	WSG		
Basic protein	Mercuric bromophenol blue (MBPB)	+	+	None	None
Tyrosine and phenols	Millon's reaction/ Diazotization	+	+	duodenum, skin	None
Tryptophan	DMAB	+	+	duodenum	Performic acid
Disulphide groups (-SS-)	Performic acid-alcian blue/DDD	+	+	skin	None
Sulphydryl groups (-SH-)	Mercury orange/DDD	+	+	skin	None
Nucleic acids	Methyl green/Pyronin	+	+	skin	None
Lipid	Oil red 0/Sudan black	+	+	skin	Chloroform-methanol extraction
Polysaccharide	PAS	+	-	liver	Acetylation
Glycogen	Best's carmine/ Diastase/PAS	+	-	liver	None
Galactogen	Pectinase/PAS	+	-	liver	None
Muco and glycoproteins	Pepsin/PAS	+	-	liver	None
Acid mucosubstances	Alcian blue/Toludine blue/Dialysed iron	+	+	skin	None

WSG - Whole salivary glands; + Satisfactory reaction product; - Not satisfactory (all tissues stained)

Table 5.2 Histochemical methods used on the salivary glands of male and female H. a. anatolicum

Substance demonstrated	Method	Process			Positive controls	Negative controls
		WSG	Sections	Pre-incubated glands		
Esterase	α-Naphthyl acetate/ Indoxyl acetate	+	+	-	Liver, kidney	NS
Aminopeptidase	Leucyl naphthylamide	+	-	-	Kidney, Intestine	NS
β-Glucuronidase	Post-coupling	+	-	-	Liver, kidney	NS
Arysulphatase	Naphthol-AS sulphate	+	-	-	None	NS
Acid phosphatase	Azo dye/lead	+	+	+	Liver, kidney	NS
Alkaline phosphatase	Azo dye/calcium	+	+	+	Liver, kidney	NS
Glucose-6-phosphatase	Lead salts	+	-	+	Liver, kidney	NS
Adenosine triphosphatase	Lead salts	+	+	+	Heart, kidney	NS
Cytochrome oxidase	Metal chelation	+	-	+	Liver, kidney	NS
DOPA-oxidase	DOPA	+	-	+	Skin	NS
Monoamine oxidase	Tetrazolium	+	-	+	Liver, kidney	NS
Succinate dehydrogenase	NBT/MTT	+	-	+	Liver, kidney	NS
NADH-diaphorase	MTT	+	-	+	Liver, kidney	NS
NADPH-diaphorase	NBT	+	-	+	Liver, kidney	NS

WSG - Whole salivary glands; + Satisfactory reaction product; - Not satisfactory;

NS - No substrate

The secretory granules of a cells of type II acini, and d and e cells of type III acini reacted moderately for basic proteins (Figure 5.1) and strongly for lipids (Figure 5.2).

Cells b and c of type II, f cells of type III, g cells of type IV acini contained secretory granules which stained strongly for polysaccharides and proteins (Figures 5.1, 5.3, 5.4 and Table 5.3). It is possible that these granules contained polysaccharide-protein complexes, presumably glycoproteins. There was considerable variation in the staining intensities of the secretory granules within and between cells. However, the secretory granules of c cells always stained more strongly than those of f and g cells (Figure 5.4 and Table 5.3). The cytoplasm of c_1 and c_3 cells also showed a weak to moderate polysaccharide activity during feeding. A progressive decrease in the numbers of PAS positive secretory granules in c_1 and c_3 was indicated by a few PAS positive granules visible in some of these cells by 72 hours of feeding(Figure 5.5). However, there was very little change in the staining intensities of secretory granules during feeding.

Pre-treatment with diastase, pectinase and pepsin did not affect the PAS staining; this suggested that the polysaccharide staining was not due to glycogen, galactogen, muco and glycoproteins. The absence of acid mucosubstances was indicated by failure to stain with Toluidine Blue, Alcian Blue or the Dialized Iron method. The secretory granules of a, b, c, d, e and f cells showed varied reactions for tyrosine, tryptophan, sulphydryl groups and ribonucleic acid (Table 5.3). A marked increase in the basic proteins and tyrosine activity of g cell granules was observed towards the later stages of feeding.

Table 5.3 Distribution of proteins, lipids and polysaccharides in the salivary glands of male and female H. a. anatolicum ticks during feeding

Acinus type	Cell type	Proteins	Tyrosine	Tryptophan	Disulphide groups (-SS-)	Sulphydryl groups (-SH-)	Nucleic acids	Lipids	Polysaccharides
Acinus I	-	2-1, c	-	1-1, c	-	1-1, c	1-1, c	3-1, c	1-0, c
Acinus II	a	2-1, g	2-2, g	-	1-0, g	3-3, g	1-1, c	3-3, g	-
	b	2-2, g	2-2, g	0-1, g	1-2, g	2-2, g	1-1, c	0	2-3, g
	c_1	1-1, g	3-2, g	1-1, g	0	2-3, g	1-2, c	0	3-4, g
	c_2	2-2, g	2-2, g	1-1, g	0	3-3, g	1-1, c	0	2-3, g
	c_3	2-1, g	2-3, g	1-1, g	2-3, g	3-3, g	1-1, c	0	4-2, g
	i	1-1, c	0	0	0	0	0	0	0
Acinus III	d	2-1, g	2-2, g	2-2, g	1-1, g	2-3, g	1-1, c	3-2, g	0
	e	2-2, g	3-4, g	1-1, g	1-1, g	4-4, g	1-1, c	2-1, g	0
	f	1-2, g	1-1, g	1-1, g	0	1-2, g	1-2, c	0	2, g
	i	1-1, c	0	0	0	0	0	0	0
Acinus IV	g	1-4, g	1-3, g	0-1, g	0	1-3, g	1-2, c	±, g	1-1, g
	i	1-1, c	0	0	0	-	0	0	0

g = reaction in secretory granules; c = reaction in the cytoplasm; i = interstitial cells;
Numbers (0-4) indicate intensity of reaction (0 = no reaction; ± = faint; 1 = weak; 2 = moderate; 3 = strong; 4 = very strong), sequence of numbers indicates increase or decrease - during feeding.

Addendum at end

5.3.1.2 Non-specific esterases: The cytoplasm of type I acini, a cells of type II, and d and e cells of type III acini showed a faint diffused reaction for non-specific esterase, which disappeared during early feeding. The secretory granules of b, c_1 and c_3 cells of type II acini showed moderate to strong non-specific esterase activity (Figures 5.6 and 5.7), the staining being more intense in c_1 and c_3 cell granules (Table 5.4). By day 3 of feeding some of the c_1 and c_3 cells had secreted most of their esterase material (Figure 5.7) and the acinar lumen was occasionally seen filled with esterase positive material. The reactions were identical in male and female ticks.

5.3.1.3 Other hydrolytic enzymes: The cytoplasm of type I and II acini was strongly positive for aminopeptidase as compared to the type III acinus. In addition the cytoplasm of the a cells (acinus II), d and e cells (acinus III) reacted moderately for β-glucuronidase. A weak sulphatase activity was also found in the cytoplasm of e cells. At the onset of feeding a progressive decrease in staining intensity of these enzymes was observed (Table 5.4).

5.3.1.4 Phosphatases: The cytoplasm of types I, II, III and IV acini showed a uniformly diffused, moderate to strong reaction for alkaline phosphatase (Figure 5.8), acid phosphatase, glucose-6-phosphatase and a faint reaction for 5-Nucleotidase and ATP-ase (Table 5.4). In general there was a slight increase in the intensity of staining for phosphatases during feeding. However, a much greater increase in ATP-ase and acid phosphatase activity was seen in the interstitial cells. The enzymic activity appeared to be concentrated

Table 5.4 Distribution of esterases, aminopeptidase, phosphatase and oxido-reductase in the salivary glands of male and female H. a. anatolicum

Acinus type	Cell type	Esterase * **	Amino-peptidase	β-glucuro-nidase	Sulphatase	Acid phospha-tase	Alkaline phospha-tase	Glucose-6-phospha-tase	ATP-ase	Cyto-chrome oxidase	DOPA-oxidase	Mono-amine oxidase	Succinate dehydro-genase	NADH diaphor-ase	NADPH diaphor-ase
I		±-0,c	3-3,c	2-1,c	±,c	2-2,c	2-1,c	1-1,c	1-0,c	1-3	2-3,c	2-2,c	3-3,c	1-1,c	2-2.c
II	a	0	3-2,c	2-1,c	±,c	4-2,c,g	2-2,c	2-2,c	±,c	1-2,c	2-2,c	2-1,c	3-2,c	1-1,c	3-3,c
	b	2-3,g	3-2,c	0	0	3-2,c	2-3,c	2-2,c	±,c	1-2,c	3-1,c,g	1-0,c	2-3,c	1-1,c	3-3,c
	c_1	3-4,g	3-2,c	0	0	2-3,c	2-3,c	2-2,c	±,c	1-2,c	3-1,c	1-1,c	2-3,c	1-1,c	3-3,c
	c_2	1-0,g	3-2,c	0	0	2-3,c	2-2,c	2-2,c	±,c,g	1-2,c	2-2,c,g	1-0,c	2-3,c	1-1,c	3-3,c
	c_3	2-3,g	3-2,c	0	0	2-3,c	2-2,c	2-2,c	±,c	1-2,c	3-2,c	1-1,c	2-3,c	1-1,c	3-3,c
	i	0	1-1,c	0	0	0-2,c	1-2,c	1-2,c	1-3,c	1-3,c	0	±-0,c	±-0,c	2-3,c	2-3,c
III	d	0	2-2,c	2-2,c	±,c	3-2,g,c	2-2,c	1-1,c	±,c	1-1,c	1-0,c	1-0,c	3-3,c	1-1,c	2-2,c
	e	0	2-3,c	2-1,c	2-1,c,g	3-3,c	2-2,c	1-1,c	±,c	1-3,c	2-0,c	1-0,c	2-4,c	1-1,c	2-3,c
	f	0	3-2,c	±,c	±,c	2-4,c	2-4,c	1-3,c	±-1,c	1-3,c	1-±,c	1-1,c	2-1,c	1-2,c	2-3,c
	i	0-1,c	2-1,c	0-1,c	0	1-4,c	1-4,c	1-2,c	1-4,c	1-3,c	-	±-1,c	1-2,c	2-3,c	2-4,c
IV	g	0	1-2,c	0	0	2-3,c	1-2,c	0-1,c	±,c	1-2,c	±,c,g	±,c	1-2,c	1-2,c	1-3,c
	i	0	1-0,c	0	0	0-1,c	±-1,c	0-1,c	±,c	0-1,c	0	-	0-1,c	0-1,c	1-2,c

c = reaction in the cytoplasm; g = reaction in secretory granules; i = interstitial cells

Numbers (0-4) indicate intensity of reaction (0 = no reaction; ± = faint; 1 = weak; 2 = moderate; 3 = strong; 4 = very strong), sequence of numbers indicates increase or decrease during feeding.

Addendum at end

in the lumen and along the lumenal border (Figures 5.9 and 5.10). The secretory granules of a cells (II acinus) and d cells (III acinus) also stained positively for acid phosphatase. Most of these granules were secreted during early feeding. The reactions varied in intensity during feeding and were similar in both sexes.

5.3.1.5 Oxidoreductases: The cytoplasm of type I and all the granular cells of types II, III and IV acini showed a diffuse to speckled reaction of oxidoreductases. The reactions were particularly strong for DOPA oxidase and NADPH diaphorase (Table 5.4). An increase in the intensity of staining for cytochrome oxidase and a decrease in the staining intensity for DOPA oxidase and monoamino-oxidase were detected during feeding (Table 5.4). In type III acini the cytoplasm of d and e cells reacted strongly for succinate dehydrogenase. In general, the enzyme activity tended to be concentrated along the acinar lumen. Reactions were similar in both sexes. The tests for succinate dehydrogenase and glucose-6-phosphatase-dehydrogenase were done on fresh tissues and the results obtained corroborated the distribution of mitochondria in types I and III acini (4.3).

5.3.2 Histochemistry of Tick Feeding Sites

The mouthparts of H. a. anatolicum penetrated deeply into the host skin. The attachment was secured by the secretion of a cone of attachment cement which diffused into adjacent collagen bundles. The secretion of cement material continued to flow into the host tissues as the lesion developed until 72 hours post attachment. Discrete aggregates of cement substances were seen away from

the main cone. However, there was no secretion of secondary cement.

The results of histochemical tests have been summarised in Table 5.5.

5.3.2.1 Proteins, lipids and polysaccharides: The cement cone of H. a. anatolicum stained intensely for basic proteins and lipids. Amongst proteins, there was a strong reaction for tyrosine, moderate for tryptophan and faint for sulphydryl and disulphide groups. The cement cone was apparently negative for polysaccharides, except for a rare streaked reaction. However, patches of glycoprotein material were occasionally seen immediately below the mouthparts and at a distance from them in tissue spaces. The homogenous collagen tissue which diffused into the cement cone gave a very faint reaction for polysaccharides. However, there were no separate zones in the cement cone. The granulocytes, chondriocytes, sebaceous glands and the affected epidermis showed moderate to strong intrinsic reactions for polysaccharides.

5.3.2.2 Non-specific esterases: A moderate non-specific esterase activity in the cement cone and in the immediate vicinity of the mouthparts was detected in cryostat sections. However, in methacrylate sections, only streaked reactions were noticed in the cone. Traces of esterase positive material were often seen in the feeding lesion. By 72 hours of feeding the cytoplasm of some neutrophils and mononuclear cells also showed a moderate to strong esterase activity indicating the uptake of this enzyme. In addition, a strong intrinsic esterase activity was observed in the hair follicles.

Table 5.5 Results of histochemical studies on the feeding sites of H. a. anatolicum.

Demonstrated	Attachment cement	Intercellular spaces at the feeding site
Basic protein	3-3 * **	0-1
Tyrosine	3-3	0
Tryptophan	1-1	0
Disulphide groups	±	0
Sulphydryl groups	±	0
Lipids	2-2	0
Polysaccharide	0	0-1
Esterase	±	0-1
Aminopeptidase	3-3	0
Sulphatases	0	0
β-Glucuronidase	0	0
Acid phosphatase	1-1	0
Alkaline phosphatase	0	0
Adenosine triphosphatase	0	0
DOPA-oxidase	0	0
Cytochrome oxidase	±	0
Monoamine oxidase	0	0
NADPH-diaphorase	0	0

Numbers (0-3) indicate intensity of reaction (0 = no reaction; ± = faint; 1 = weak; 2 = moderate; 3 = strong), sequence of numbers indicates increase or decrease during feeding.

Addendum at end

5.3.2.3 Aminopeptidase: A moderate to strong aminopeptidase activity, highly localized in the cement cone, was observed (Table 5.5). The hair follicle and the hair shaft also gave positive reactions. The cytoplasm of a few mononuclear cells showed a faint reaction during later stages of feeding.

5.3.2.4 Phosphatases: The cement cone was negative for alkaline phosphatase and ATP-ase, and gave a faint streaked reaction for acid phosphatase (Table 5.5).

In addition to a weak generalised cytoplasmic reaction in all cells, there was a strong reaction in the hair follicles and sebaceous glands for acid and alkaline phosphatases.

5.3.2.5 Oxidoreductases: The cement cone did not show any activity for any of the oxidoreductases. However, very strong intrinsic reactions were observed in the inflammatory cells.

5.4 DISCUSSION

The structural changes in the salivary glands in relation to their function have been discussed in Chapter 4 and will not be repeated.

The histochemical observations on the presence and distribution of lipids, proteins and polysaccharides in type I acini corroborated those of Coons and Roshdy (1973), Binnington (1978) and Walker et al. (1984). The lipid and glycogen component in the cytoplasm of type I acini might act as an energy reserve during non-parasitic stages. In addition the lipid droplets could well be the energy reserves for accumulating chloride ions (Coons and Roshdy, 1979) associated

with sodium and potassium ions in oral secretions during questing stages (Rudolph and Knulle, 1978).

A rapid reduction in the lipid and glycogen contents and increased activity of glucose-6-phosphate dehydrogenase, cytochrome oxidase and DOPA oxidase might suggest that the excretion during feeding of salts was no longer required. An increased level of cyclic AMP (Krolak et al., 1983) during feeding was further suggestive of such a function.

The presence of a basal labyrinth richly supplied with mitochondria (Figures 4.5 and 4.9) and the presence of ATP-ase supported the hypothesis of McMullen et al. (1976) that the type I acini were responsible for the production of hygroscopic saliva to absorb water vapours from an unsaturated atmosphere.

The physiological significance of non-specific esterase and β-glucuronidase is not clear.

The histochemical results further showed that the a cells of type II acinus, and d and e cells of type III acinus were the cement precursor cells in H. a. anatolicum. The attachment cement stained for lipoproteins in much the same way as did the secretory granules of a, d and e cells. In common with the secretory granules of these cells the cement cone was rich in tyrosine and also showed a weak reaction for tryptophan. The a, d and e cells were filled with secretory granules before attachment and the timing of their secretion corresponded with the timing of cement deposition. Although it is difficult to assign individual functions to these cells, it is possible that the secretions of a cells of acini II formed the main cone whilst

the d and e secreted material thereafter into the feeding lesion to compensate collagen destruction due to the cytolytic effect of salivary secretions or the host response. Cells analogous to a, d and e with similar histochemical properties have been suggested as the cement precursor cells in H. spinigera (Chinery, 1973); B. microplus (Binnington, 1978) and R. appendiculatus (Walker et al., 1984).

In contrast to B. microplus (Moorhouse and Tatchell, 1966) and H. spinigera (Chinery, 1973) the attachment cement of H. a. anatolicum was homogenous in texture and did not show distinct internum and cortex. These differences in the formation of the cement cone might reflect their different attachment patterns. Unlike B. microplus and like H. spinigera the cement cone of H. a. anatolicum was negative for polysaccharides.

There was no evidence to support the hypothesis of Moorhouse and Tatchell (1966) that the cement was hardened by quinone tanning of disulphide bonds, as the cement cone stained very weakly for sulphydryl and disulphide groups and was negative for DOPA-oxidases.

Like R. sanguineus (Theis and Budwiser, 1974) there was no evidence for the secretion of a secondary cement just before or during the rapid engorgement phase as suggested for B. microplus (Tatchell and Moorhouse, 1968). The cement precursor cells in H. a. anatolicum were almost inactive by 72 hours post-attachment.

The secretory granules of b and c cells of type II acini were rich in glycoproteins and non-specific esterases. Cells with similar histochemical properties have been found in type II acini of B. microplus (Binnington, 1978) and R. appendiculatus (Binnington et al.,

1983; Walker et al., 1984). Unlike R. appendiculatus (Walker et al., 1984) the glycoprotein and non-specific esterase in H. a. anatolicum appeared to be closely related. Apart from a rare, weak contamination, the cement cone was negative for glycoproteins and non-specific esterases. Patches of glycoproteins and esterase materials found at tick feeding sites might have originated from b/c cells, but they could also have come from type III acini, the cytoplasm of which showed a faint reaction before attachment. These results are consistent with the observations of Schleger and Lincoln (1976) who demonstrated esterases, aminopeptidase and lipase at larval B. microplus feeding sites. The secretion of esterase into the feeding lesion and its rapid removal in animals which rejected ticks suggested its important role for successful feeding. Esterase might increase vascular permeability by its direct action on mast-cells (Geczy et al., 1971), or by inducing the formation of plasma kinins (Movat, 1971).

A strong homogenous reaction for aminopeptidase and a weak reaction for acid phosphatase and non-specific esterase in the cement cone suggested that these enzymes were secreted as active constituents of saliva. It was impossible to determine the precise origin of aminopeptidase as the cytoplasm of all the acini showed a moderate to strong reaction for aminopeptidase. However, acid phosphatase might have originated from a cells of type II acini and d cells of type III acini. It seemed likely that the aminopeptidase was responsible for the lysis of collagen tissue adjacent to the cement cone.

From the histochemical nature of the cement cone it is most likely that it would stimulate a foreign body type reaction and would

also be antigenic following subsequent infestations. This is further supported by the isolation of antigenic esterases and aminopeptidase from the saliva of H. a. anatolicum (6.3). However, this is in contrast to the observations of Tatchell and Moorhouse (1968) that the attachment cement of B. microplus was inert.

Non-specific esterases and aminopeptidases are weakly hydrolytic enzymes. They might be responsible for creating, over a period of days, considerable tissue damage to provide the tick with its blood meal. Weakly hydrolytic enzymes have also been demonstrated in the salivary glands of R. appendiculatus (Martin, 1977; Walker et al., 1984) and B. microplus (Binnington, 1978).

It was interesting that acid mucosubstances were absent from the salivary glands of H. a. anatolicum. According to Balashov (1972) salivary anticoagulants were polysaccharide-protein complexes. So if the anticoagulants were present in the salivary glands of H. a. anatolicum then the glycoprotein secretions of b and c cells of type II (especially metachromatic granules of c_2) and transitory secretions of f cells of type III acini could be the possible source of this activity.

The cells b and c of type II acini synthesize and secrete their products throughout feeding and could be the potential sources of pharmacologically active substances (Dickinson et al., 1976; Higgs et al., 1976; Shemesh et al., 1979), proteolytic enzyme inhibitors (Willadsen and Riding, 1980), immunosuppressors (Wikel, 1981) and blockers of histamine-like activity (Chinery and Ayitey-Smith, 1977).

Moderate to strong reactions observed for phosphatases and oxidoreductases in the cytoplasm of the granular cells of types II, III

and IV acini suggested active metabolic activity in these cells. Interestingly, none of these enzymes (except acid phosphatase) appeared to be secreted into the tick feeding lesions as an active component of saliva. The rare presence of some of these enzymes in the cement cone or feeding lesion (Table 5.4) could be due to accidental contamination of the saliva by these metabolic enzymes.

Consistently strong reactions for ATP-ase and acid phosphatases along the interstitial cells and the lumenal border suggested an active transport function, as proposed by Meredith and Kaufman (1973) and Megaw and Beadle (1979). A similar distribution of these enzymes was also found by Walker et al. (1984) in R. appendiculatus.

The glycoprotein secretions of g cells of type IV acini probably ensured smooth transfer of spermatozoa during copulation as suggested by Feldman-Muhsam et al. (1970).

5.5 SUMMARY

A study was made of the histochemistry of the salivary glands and tick feeding sites of H. a. anatolicum during feeding. The attachment cement of H. a. anatolicum was lipoprotein in nature and appeared to have been derived from a cells of type II, and d and e cells of type III acini, the secretory granules of which had similar histochemical properties. Deposits of glycoprotein materials were located at tick feeding sites in dermal tissues adjacent to the mouthparts and not in the attachment cement. This polysaccharide material was presumed to be secreted by b and c cells of type II acini. Their possible role in feeding is discussed.

A strong aminopeptidase and a moderate acid phosphatase activity was found localised in the cement cone and not in the tissues. Blebs of non-specific esterase material, probably derived from *b*, c_1 and c_3 cells of type II acini were demonstrated at tick feeding sites adjacent to the mouthparts. It is presumed that the aminopeptidase might be responsible for homogenization of the collagen bundles observed along the cement cone, whereas the esterase might increase vascular permeability in naive hosts by its direct action on mast-cells or by the formation of plasma kinins.

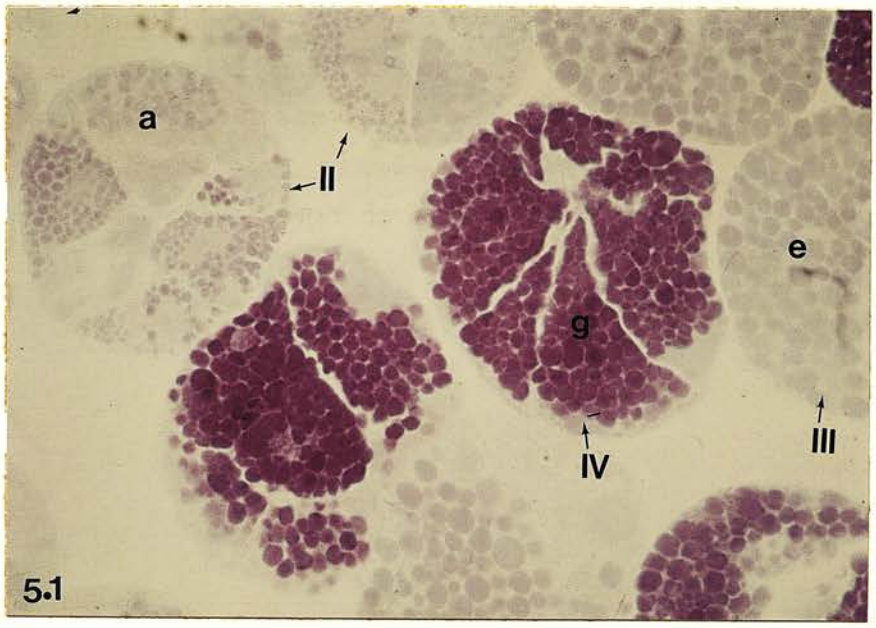
Despite the strong reactions for several oxidoreductases and other phosphatases in the salivary glands of *H. a. anatolicum*, no activity for these enzymes was detected in the cement or at feeding sites.

Figure 5.1 Acini types II, III and IV; ♂ male, 144 hours after attachment. Note a general reaction for proteins in different granular cells, particularly strong in g cells of type IV acinus. Methacrylate section (Mercuric bromophenol blue method, x 208).

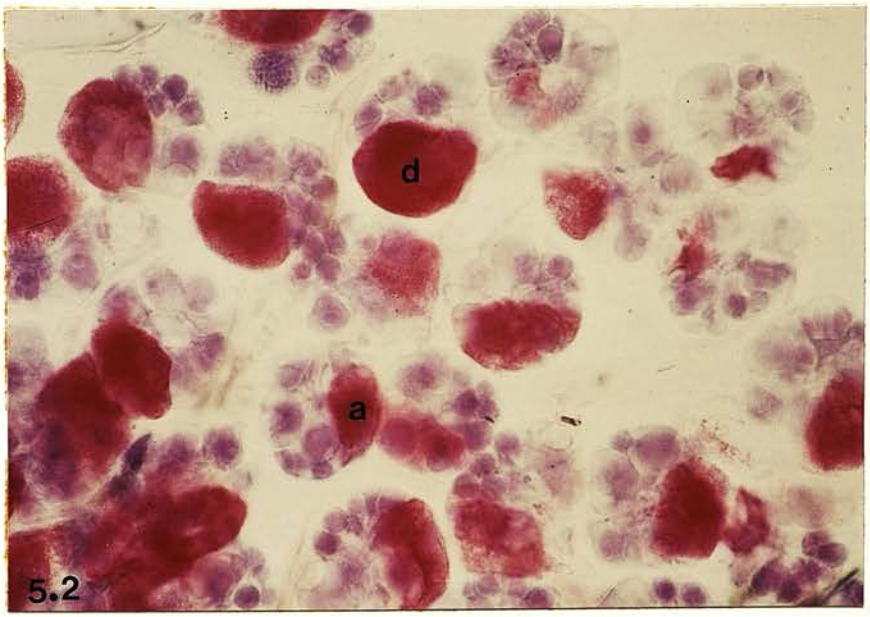
Figure 5.2 Whole salivary gland of an unfed female showing a strong lipid reaction in a cells of acinus II and d cells of acinus III (Oil red 0 method, x 104).

g = g cells; a = a cells; d = d cells;

e = e cells; sg = secretory granules.



5.1



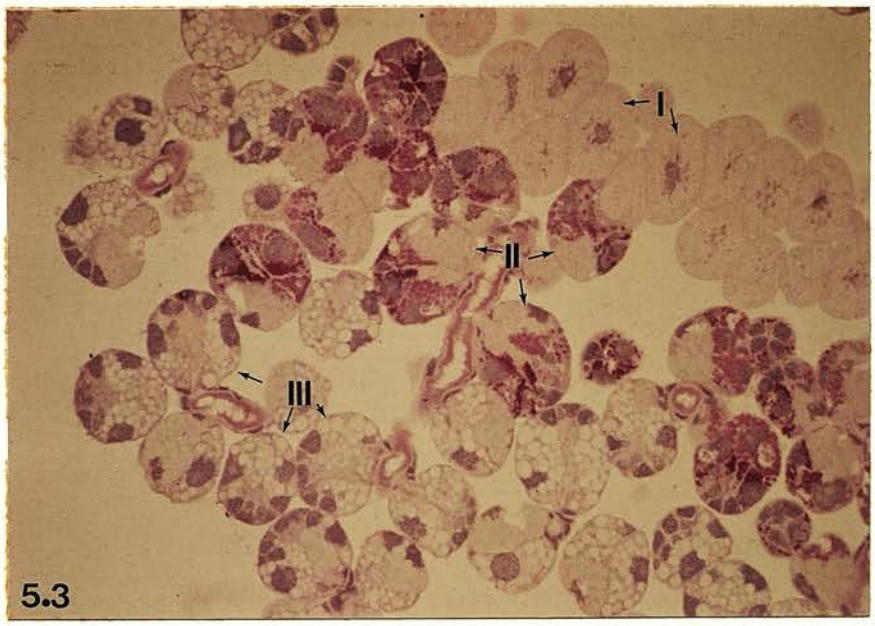
5.2

Figure 5.3 Acini types I, II and III of an unfed female showing a speckled reaction for polysaccharides in type I acinus and moderate to strong reaction in granules of b and c cells of type II acinus. Methacrylate section (PAS reaction, x 104).

Figure 5.4 Acini types I, II, III and IV of a 144 hour fed male showing a strong polysaccharide reaction in granules of b and c cells of acinus II and a moderate reaction in granules of g cells. Methacrylate section (PAS reaction, x 208).

b = b cells; c = c cells; g = g cells;

sg = secretory granules.



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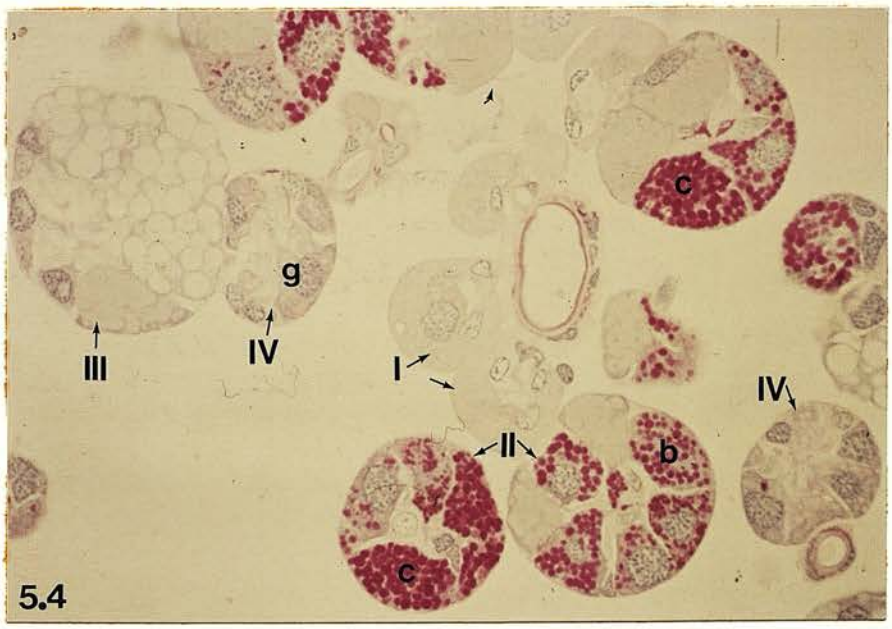


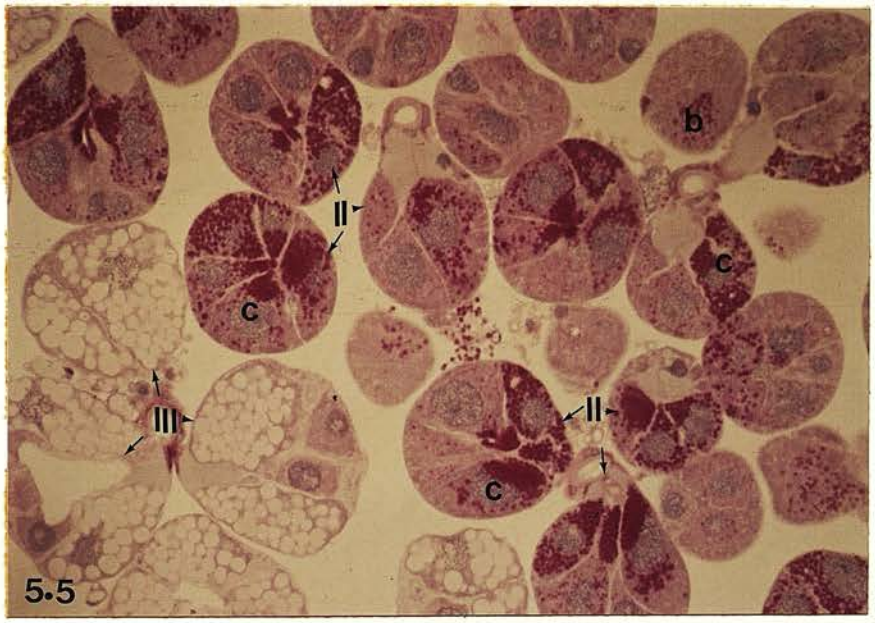
5.4

Figure 5.5 Acini types II and III of a 72 hour fed female, showing polysaccharide granules crowded towards acinar lumen of acinus II and certain b and c cells of acinus II with very few polysaccharide granules left in them. Methacrylate section (PAS reaction, x 104).

Figure 5.6 Acini types I, II and III of an unfed female showing strong esterase activity in c cells and weak activity in b cells of acinus II. Methacrylate section (α-Naphthyl acetate method, x 104).

b = b cells; c = c cells;

sg = secretory granules.


b
II
c
c
III
II
c
5.5

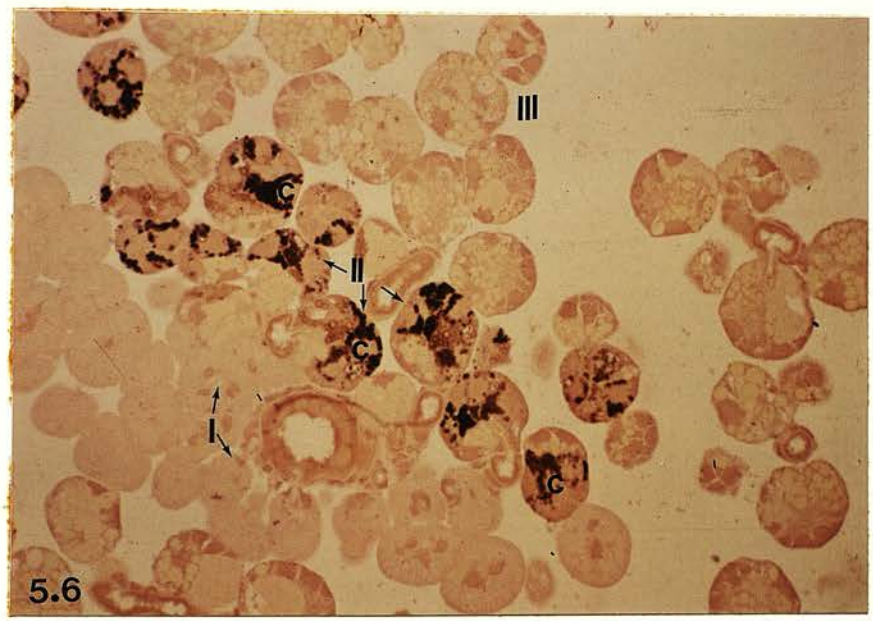
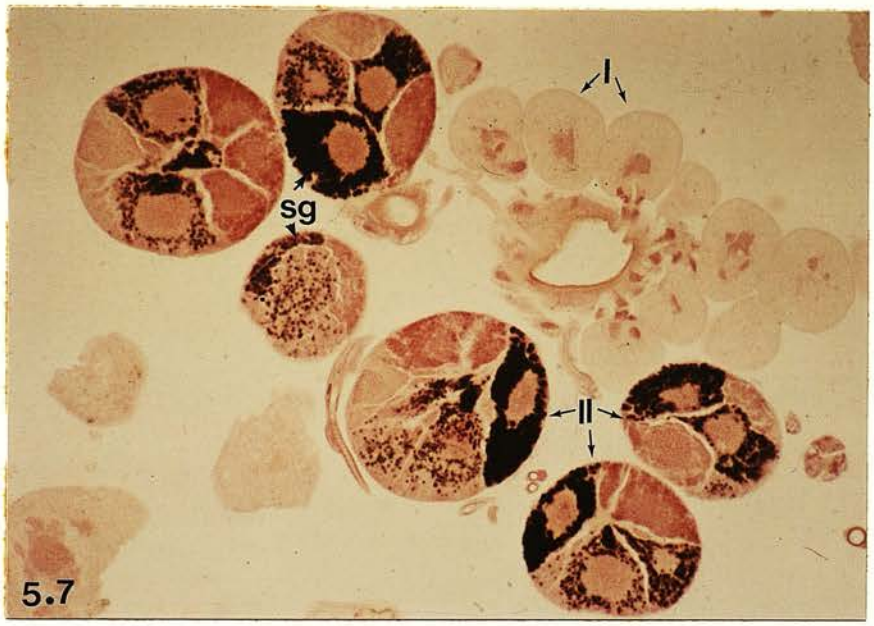

III
c
II
c
I
c
5.6

Figure 5.7 Acini types I and II of a 144 hour fed male, showing strong esterase activity in c cell types of acinus II. Methacrylate section (α-Naphthyl acetate method, x 208).

Figure 5.8 Whole salivary gland of a 72 hour fed female, showing diffused alkaline phosphatase activity in the cytoplasm of acini types II and III. (Azo dye coupling method, x 104).

c = c cells; sg = secretory granules.


I
sg
II
5.7

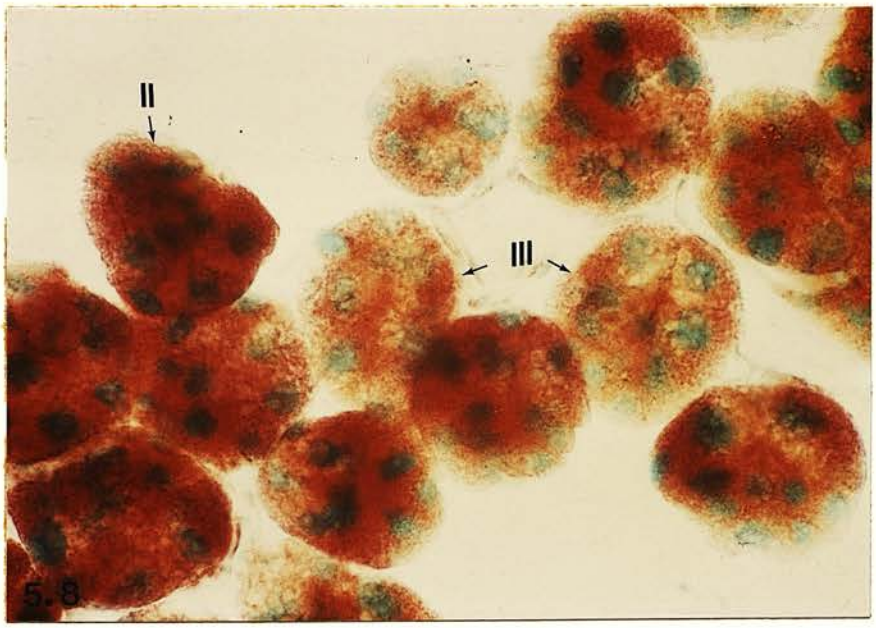
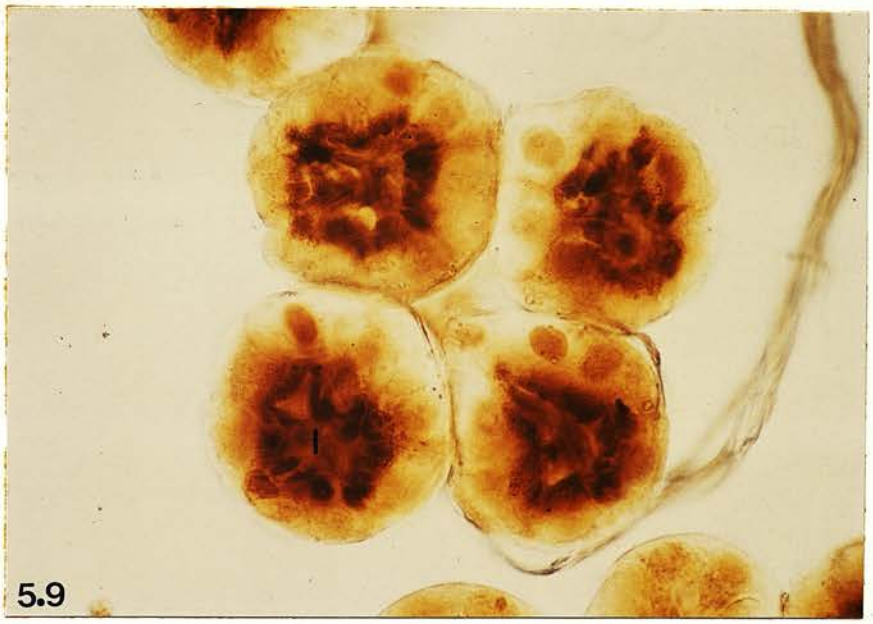

II
III
5.8

Figure 5.9 Whole salivary gland of a 72 hour fed female, showing speckled adenosine triphosphatase activity along the acinar lumen of type III acinus. (Lead method, x 208).

Figure 5.10 Whole salivary gland of a 72 hour fed female, showing strong acid phosphatase activity along the acinar lumen of acini types II and III and mild in cytoplasm of type I acinus. (Gomori lead method, x 104).

l = lumen.



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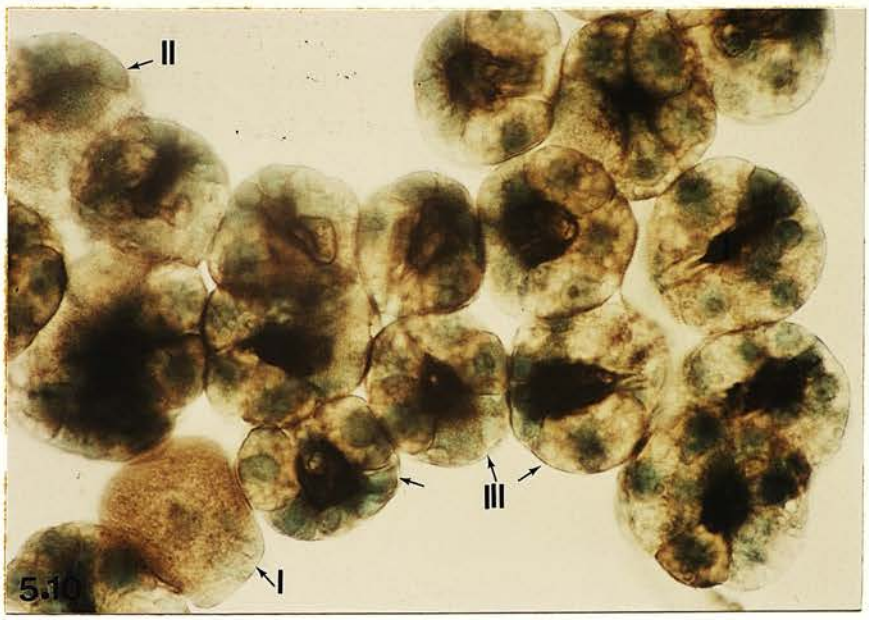


5.10

CHAPTER SIX

ISOLATION AND CHARACTERIZATION

OF SALIVARY ANTIGENS

CONTENTS

6.1 INTRODUCTION

Despite a wealth of information on the mechanisms of the immune response, the nature of antigens involved in the induction of resistance has received very little attention. Attempts to artificially immunise potential hosts against ticks using crude antigens have to date met with limited success. Antigens used in these studies have included reproductive organs, gut tissues (Allen and Humphreys,1979), extracts of whole ticks (McGowan et al., 1981) and salivary glands (Wikel, 1981). The results of these studies indicate that the most effective antigens originate from the salivary glands. The result is perhaps not surprising as the salivary glands of ixodid ticks have been shown to be a source of hydrolytic enzymes (Tatchell, 1971), anticoagulants (Ross, 1926; Kaire, 1967) and other pharmacologically active substances (Dickinson et al., 1976; Shemesh et al., 1979). Glycoproteins, lipoproteins and several enzymes have also been histochemically demonstrated in salivary glands (Binnington, 1978; Gill and Walker, 1984; Walker et al., 1984) and at tick feeding sites (Schleger and Lincoln, 1976; 5.3). It therefore seems likely that many of these components could stimulate a host response detrimental to the tick. However, the evidence for this is still lacking. Therefore, the objectives of the study described in this chapter were to isolate and characterize the antigens originating from H. a. anatolicum salivary glands.

6.2 EXPERIMENTAL DESIGN

Salivary gland extracts (SGE) from unfed female ticks and ticks fed for 24, 96 and 144 hours were analysed by SDS-PAGE on 7% acrylamide

gel slabs to select a representative stage from which to collect saliva in order to isolate and characterize the antigenic proteins. To identify salivary antigens, saliva and SGE from females fed for 96 hours were separated as above (SDS-PAGE) and were electrophoretically transferred onto a nitrocellular paper. The resulting electroblots were incubated with specific anti-tick serum from hypersensitized rabbits. Antigen-antibody reactions thus produced were visualized either indirectly by autoradiography after incubation with ^{125}I-labelled goat anti-rabbit IgG or directly after incubation with peroxidase-conjugated goat anti-rabbit IgG (GARP) followed by appropriate substrate. The following controls were included in these experiments: (i) normal serum instead of immune serum; (ii) omission of first antibody; (iii) omission of second antibody and (iv) omission of both the first and the second antibody.

Antigens were further characterized by staining gels for glycoproteins, non-specific esterase, aminopeptidase and acid phosphatase.

Major antigens were isolated from saliva after fractionation by SDS-PAGE and were used for skin testing in hypersensitized rabbits to determine the nature of the immune response which they stimulated. Unfractionated saliva, SGE and negative controls (PBS, PBS + acrylamide) were included in the skin test for comparison. Rabbits were killed two hours and 48 hours post inoculation and the inoculation sites removed for histological examination. Tissues were fixed in Karnovsky's fixative, embedded in paraffin wax, and 5 μm thick sections cut. The sections were stained with acidic Giemsa stain for conventional histological examination. Paraffin embedding was chosen because it was rapid and produced resolution adequate for identification of the nature of the cellular infiltrate.

6.3 RESULTS

A major problem encountered in these experiments was the small amount of saliva and thus protein obtained from each tick. This problem was further increased because the sample preparation for SDS-PAGE necessitated diluting the saliva (1:1, v/v) with SDS-sample buffer. Initial attempts to overcome this by using thin gels (0.7 mm) together with small sample volumes were unsuccessful. They were abandoned in favour of thick gels (1.5 mm) and large sample volumes (150 µl).

Another difficulty encountered was that the proteins separated on SDS-PAGE could not be stained for enzymes to help characterize individual antigens. To overcome this problem fractionation was carried out using agarose IEF and non-SDS polyacrylamide gel electrophoresis. The results presented in this study are from four replicate experiments.

6.3.1 SDS-PAGE Separation of Saliva and Salivary Gland Proteins

In the gland extracts examined, the least number of protein bands were found in unfed ticks (20), but the number of protein bands increased in 24 hour fed (32), reached a maximum in 96 hour fed (45), before falling (39) in 144 hour fed females (Figure 6.1). The total amount of protein present in the extracts was lowest in the unfed and highest in the 96 hour fed female ticks. A total of 19 protein bands were detected in saliva obtained from 96 hour fed females but one band which had an apparent molecular weight of 96,000 daltons constituted the bulk of salivary proteins (Figure 6.1, track E). Another band having a molecular weight of 130,000 daltons was the most prominent

protein band in saliva. Interestingly, from the 19 proteins identified in tick saliva, 14 were common to all the stages examined.

6.3.2 Identification of Salivary Antigens

The high resolving power of SDS-PAGE together with the high sensitivity of immunochemical and autoradiographic techniques produce an extremely powerful qualitative tool for studying antigen-antibody interactions. Sera from hypersensitized rabbits recognised nine antigens in the saliva and 17 in the SGE from females fed for 96 hours (Figures 6.2 and 6.3). A comparison of two techniques (peroxidase/^{125}I-labelled goat anti-rabbit IgG) used to visualize antigens did not reveal any differences in either the position or the number of antigens (Figures 6.2 and 6.3). Three antigens with molecular weights of 60,000, 94,000 and 96,000 daltons reacted very weakly with a normal rabbit serum control. All the antigens detected in the saliva were found in the SGE of unfed, 24, 96 and 144 hour fed female ticks.

6.3.3 Characterization of Salivary Antigens

All nine antigens detected in the saliva from 96 hour fed females stained as glycoproteins (Figure 6.4). A comparison of the two methods used to detect glycoproteins showed that the Concavalin A-peroxidase method gave better results than the Schiff's method.

Separation of the saliva using agarose IEF revealed only one major protein band which had an isoelectric point of pH 6.15 (Figure 6.5) and corresponded to antigen III separated by SDS-PAGE. The protein band stained strongly for non-specific esterase activity (Figure 6.5) and weakly for aminopeptidase.

Acid phosphatase activity was found to be associated with the 130,000 dalton, molecular weight protein band (antigen I), present in both saliva and SGE of 96 hour fed females (Figure 6.6).

6.3.4 Skin Reactivity to Salivary Antigens

The three major antigens found in saliva (Antigen I, molecular weight 130,000; Antigen II, molecular weight 103,000; Antigen III, molecular weight 96,000) were isolated after SDS-PAGE and inoculated intradermally into four hypersensitized rabbits to test their ability to induce specific allergic responses. Unfractionated saliva and SGE from 96 hour fed females were included for skin tests along with negative controls (PBS and PBS + acrylamide).

The skin reactivity of hypersensitized rabbits to salivary antigens is presented in Figure 6.7. All five antigens tested elicited a moderate degree of immediate skin reactivity. The reaction sites were oedematous, hyperaemic and raised above the skin. These reactions became apparent 15 minutes post inoculation, peaked after two hours and then decreased slightly in diameter by four hours. Three of the antigens: antigen III, saliva and SGE produced similar but less intense skin reactions in a control rabbit.

In addition to immediate skin reactions, all the antigens, except antigen I, induced strong delayed skin reactivity when compared to similar sites on a naive rabbit and to control sites on sensitized rabbits. The reaction sites were oedematous, indurated and slightly necrotic at 48 hours. The reactions elicited by antigen III, saliva and SGE were comparatively more intense than those elicited by antigen II. These reactions reached a maximum at 24 hours and

decreased from that time onwards, but were still quite prominent at 48 hours.

6.3.5 Histology of Inoculation Sites

Histological examination of skin biopsies taken from inoculation sites on sensitized hosts two hours post inoculation revealed a large number of eosinophils and few neutrophils. A few of the mast-cells exhibited degranulation. Blood vessels in the vicinity of reaction sites exhibited increased permeability, as indicated by oedema and extravasation of inflammatory cells.

In biopsies taken from sensitized hosts 48 hours post inoculation, the inflammatory infiltrate was dominated by neutrophils and mononuclear cells. Eosinophils and basophils were present in small numbers. Perivascular cuffing by mononuclear cells was a constant feature. Blood vessels were dilated and engorged with inflammatory cells. Endothelial cells appeared swollen and protruded into the vessel lumen. There was marked oedema, resulting in overall thickening of the dermis.

The cellular infiltrate at control sites on sensitized hosts was insignificant as compared to the reaction to salivary antigens and consisted mainly of neutrophils. The inoculation sites of salivary antigen in controls had few neutrophils.

6.4 DISCUSSION

SDS-PAGE separation of saliva and salivary gland extracts showed the presence of a large number of salivary proteins. The progressive increase in the numbers and concentration of protein bands

present in the salivary glands during feeding, reaching a maximum at 96 hours and then decreasing slightly by 144 hours (Figure 6.1), suggested an active synthesis and secretion of salivary components, probably to meet the increased physiological requirements during feeding. The pattern of protein changes in the salivary glands observed during feeding was similar to that reported by McSwain et al. (1982) for female A. americanum ticks. The changes observed in the protein profiles of salivary gland extracts from different feeding stages corresponded with the histochemical changes found in the salivary glands of H. a. anatolicum (5.3). The maximum number of protein bands at 96 hours (Figure 6.1) further coincided with the peak granular activity of b and c cells of type II acini (4.3 and 5.3).

Nine of the 19 protein bands recognised in the saliva were antigenic (Figures 6.2 and 6.3). The antigen-antibody complexes formed by salivary antigens were visualised by the use of either enzyme or radio-labelled goat anti-rabbit IgG. Therefore, if other proteins (antigens) stimulated the production of immunoglobulins, other than IgG,or cell mediated responses, they may not be identified by this technique. The presence of other antigens in the saliva cannot therefore be discounted.

All the salivary antigens were glycoproteins. In addition, antigen I showed acid phosphatase activity and antigen III showed both non-specific esterase and aminopeptidase activity. These results are in agreement with Tatchell (1971) who demonstrated esterase, aminopeptidase and acid phosphatase in the saliva of B. microplus ticks.

The complex nature of antigen III indicates that it is probably composed of two different proteins with very similar molecular weights as is evident in SGE. However, it is not possible to say which activity is associated with which protein. It is thought that the bulk of esterase component of antigen III had originated from b and c cells of type II acinus (5.3.1.2). The aminopeptidase activity might have originated from other cells of types I, II and III acinus (5.3.1.3).

Owing to the antigenic nature of these enzymes it would appear that they are secreted into the host during feeding and might perform important functions. The localization of these enzymes at tick feeding sites (5.3.2) and the rapid removal of esterases from hosts which rejected ticks (Tracey-Patte, 1979) suggested the essential role of these enzymes for successful feeding. Of the three allergens isolated from B. microplus larvae two are known to have other important physiological functions (Willadsen and Williams, 1976; Willadsen and Riding, 1980).

The presence of antibodies against nine salivary proteins, which together formed the bulk of the salivary proteins, suggested one of a number of possible effector mechanisms by which the mammalian hosts might express resistance against ticks. It is likely that the majority of the salivary proteins interact with host antibodies to form immune complexes either at the feeding site or in the gut of the tick (or both) and might deprive the tick of important digestive enzymes. However, this would largely depend upon the antigen - antibody proportions to ensure sufficient complex formation to reduce feeding.

Whether the binding of antibody to antigen impairs the physiological activity of the antigen is not known. In addition IgG antibodies might be involved in the recruitment of basophils (Brown et al., 1982b), the major effector cells of resistance against ticks (Brown et al., 1982a).

All the antigens tested (antigens I, II, III, whole saliva and SGE) elicited a moderate degree of immediate skin reactivity. This could be due to the mediators released during interactions between salivary antigens and homocytotropic antibody bound to the sensitized cells (Frick, 1982). Comparable skin reactivity was produced by antigen III, whole saliva and SGE. This was consistent with the findings of Willadsen and Williams (1976), Willadsen et al. (1978) and Willadsen and Riding (1979) who observed similar immediate oedematous skin reactions on intradermal inoculation of purified allergens from B. microplus larvae into resistant cattle. Riek (1956) equated hypersensitivity with resistance and Willadsen et al. (1979) demonstrated a significant correlation between the level of resistance and the intensity of immediate hypersensitivity to allergens purified from larval B. microplus extracts. This also corroborated the development of tick antigen reactive homocytotropic antibodies to tick infestations reported by Brossard and Girardin (1979), Boese (1974) and McGowan et al. (1979).

Immediate hypersensitivity reactions result in the release of vasoactive amines, chiefly histamine (Wells and Eyere, 1972), which has been found to have a direct effect on the attachment (Kemp and Bourne, 1980) and feeding behaviour of ticks (Paine et al., 1983).

Increased levels of histamine in the blood (Riek, 1962) and skin of resistant hosts (Wikel, 1982) have also been shown to correlate with both the level of resistance and the degree of immediate hypersensitivity.

Histological examination of immediate reaction sites showed mast-cell degranulation and eosinophil infiltration, the cellular infiltrate characteristic of immediate hypersensitivity reactions. Mediators released by degranulating mast-cells might stimulate host grooming (Schleger et al., 1976), another effector mechanism of tick rejection (Koudstaal et al., 1978).

Similar, but less intense reactivity to antigen III, saliva and salivary gland extracts observed in the control rabbits suggested a vasoactive property of H. a. anatolicum saliva. This was consistent with the findings of Tatchell and Binnington (1973) who observed increased capillary permeability in unexposed cattle on intradermal inoculation of saliva from B. microplus. Increased vascular permeability might have resulted from the salivary esterases (Geczy et al., 1971; Movat, 1971). Alternatively it might have been caused by pharmacologically active substances present in the salivary glands of ixodid ticks (Dickinson et al., 1976; Higgs et al., 1976; Shemesh et al., 1979).

All the antigens tested except antigen I, elicited moderate to strong delayed skin reactivity. The reactivity was most pronounced at the inoculation site of whole saliva, SGE and antigen III. Similar delayed skin reactivity to D. andersoni salivary gland antigen was demonstrated in tick resistant guinea-pigs (Wikel et al., 1978) and cattle (Wikel and Osburn, 1982). In addition the peripheral blood

lymphocytes and lymph node cells obtained from these hosts displayed antigen-specific lymphocyte blastogenesis. In contrast, the allergens isolated from larval extracts of B. microplus failed to elicit any delayed skin reactivity when inoculated intradermally into tick-exposed cattle (Willadsen and Williams, 1976; Willadsen et al., 1978; Willadsen and Riding, 1979). Delayed skin reactivity has been considered as the prototype of cell mediated reactivity (Jaffer et al., 1973). Resistance to D. andersoni (Wikel and Allen, 1976) and A. americanum (Brown, 1982) was successfully transferred with viable lymph node cells and sensitized peritoneal exudate cells, respectively.

Peak skin reactivity observed at 24 hours post inoculation, coincided with the massive infiltration and degranulation of basophilic leucocytes at H. a. anatolicum feeding sites in rabbits (7.3) and cattle (8.3). Basophils have been shown to be the major effectors of resistance in the guinea-pig-tick system (Brown et al., 1982a).

Histologically, the delayed reactions at 48 hours consisted of neutrophils and mononuclear cells. The perivascular cuffing by mononuclear cells and other vascular changes were typical of a delayed hypersensitivity reaction (Dvorak et al., 1974).

6.5 SUMMARY

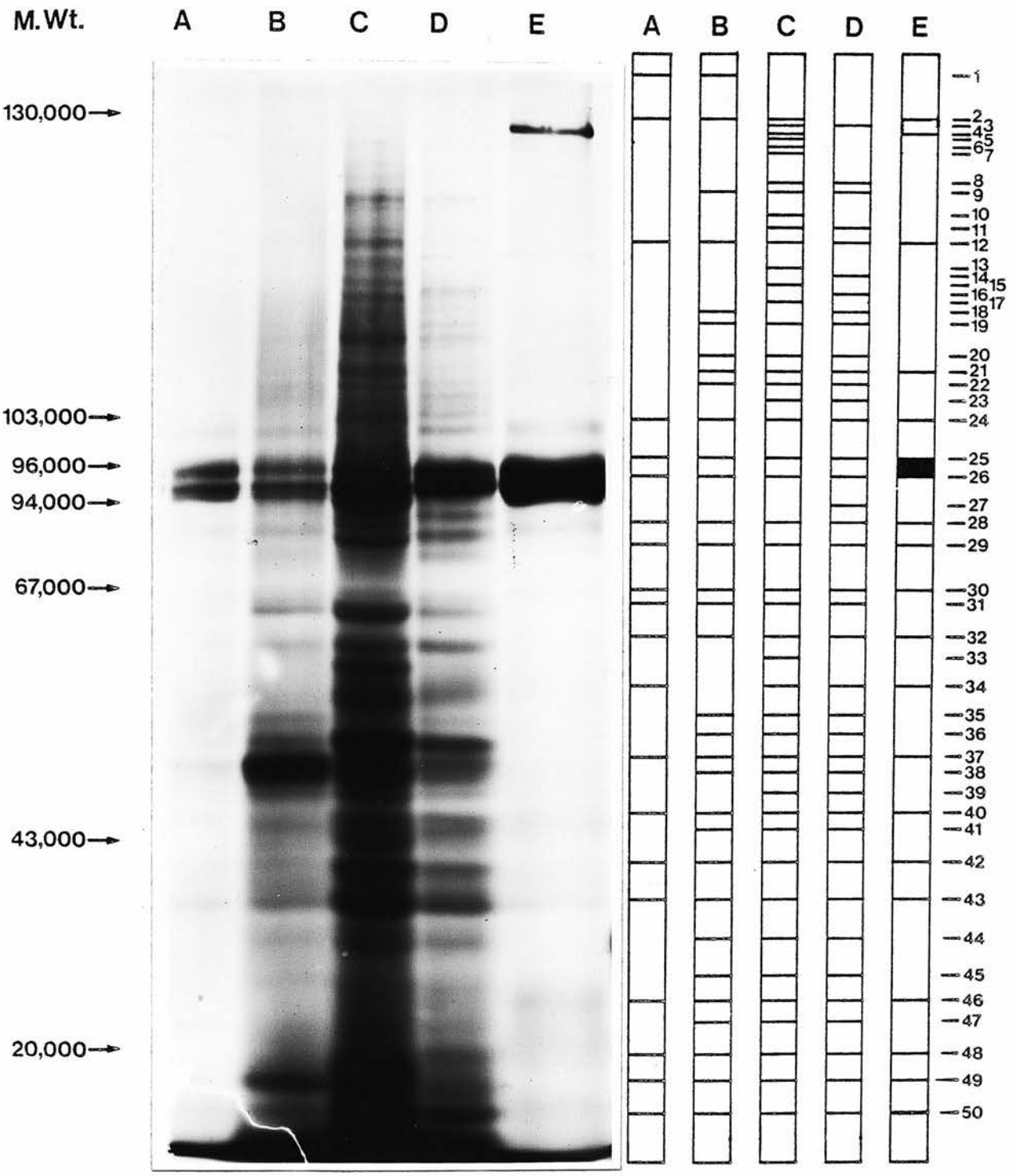
This study showed for the first time the multitude of salivary antigens involved in the acquisition of resistance to ixodid ticks. Sera from hypersensitized rabbits identified nine antigenic proteins in the saliva of 96 hour fed female H. a. anatolicum. Antigenic proteins ranged in molecular weight from 14,400 to 130,000 daltons.

All the salivary antigens were glycoprotein in nature and appeared to be common to the different stages of feeding. In addition to their glycoprotein nature, antigen I (molecular weight 130,000 daltons) showed acid phosphatase and antigen III (molecular weight 96,000 daltons) showed both non-specific esterase and aminopeptidase activity.

Three major antigenic proteins, isolated from saliva (antigen I, antigen II - molecular weight 103,000 daltons and antigen III), gave immediate hypersensitivity reactions on intradermal inoculation into rabbits which had previously been exposed to ticks. Antigen II and antigen III also elicited strong delayed hypersensitivity reactions which peaked at 24 hours post inoculation. These results help to elucidate the nature of immune mechanisms which effect resistance against H. a. anatolicum.

Figure 6.1 Protein profile of saliva and salivary gland extracts of H. a. anatolicum separated by SDS-PAGE on 7% acrylamide gel slabs. Track A, salivary gland extracts (SGE) from unfed female ticks; Track B, SGE from 1 day fed females; Track C, SGE from 96 hour fed females; Track D, SGE from 144 hour fed females; Track E, saliva from 96 hour fed females. Numbers on left represent molecular weight standards. The gel was stained with silver stain by the method of Morrissey (1981)

6.1


M.Wt.
A
B
C
D
E
130,000
103,000
96,000
94,000
67,000
43,000
20,000
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
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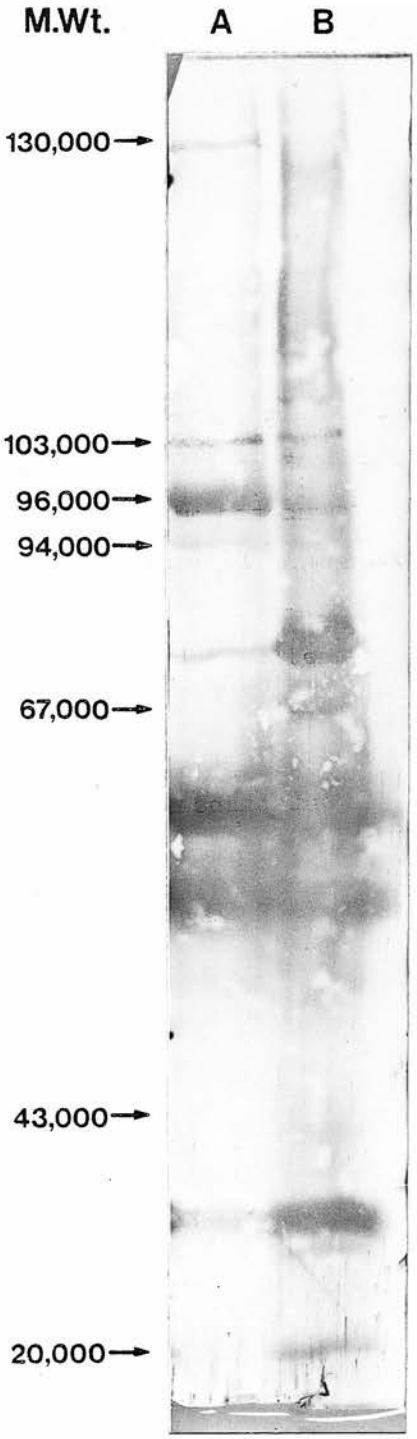
49
50

Figure 6.2 Pattern of salivary antigens of H. a. anatolicum separated by SDS-PAGE on 7% acrylamide gel slabs, electroblotted, incubated with serum from hypersensitized rabbits and developed with peroxidase conjugated goat anti-rabbit immunoglobulin (IgG) (GARP). Track A, saliva from 96 hour fed female ticks; Track B, salivary gland extracts from 96 hour fed female ticks. Numbers on left represent molecular weight standards.

6.2


M.Wt.
A
B
130,000
103,000
96,000
94,000
67,000
43,000
20,000

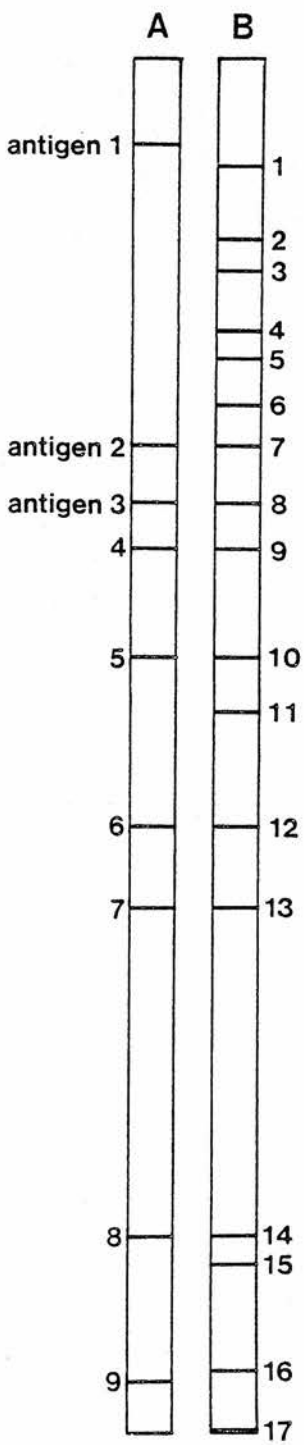

A
B
antigen 1
antigen 2
antigen 3
4
5
6
7
8
9
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17

Figure 6.3 Pattern of salivary antigens of H. a. anatolicum separated by SDS-PAGE on 7% acrylamide gel slabs, electroblotted, incubated with serum from hypersensitized rabbits, and developed with ^{125}I-labelled goat anti-rabbit immunoglobulin (IgG). Track A, saliva from 96 hour fed females; Track B, salivary gland extracts from 96 hour fed females. Numbers on left represent the molecular weight standards.

6.3

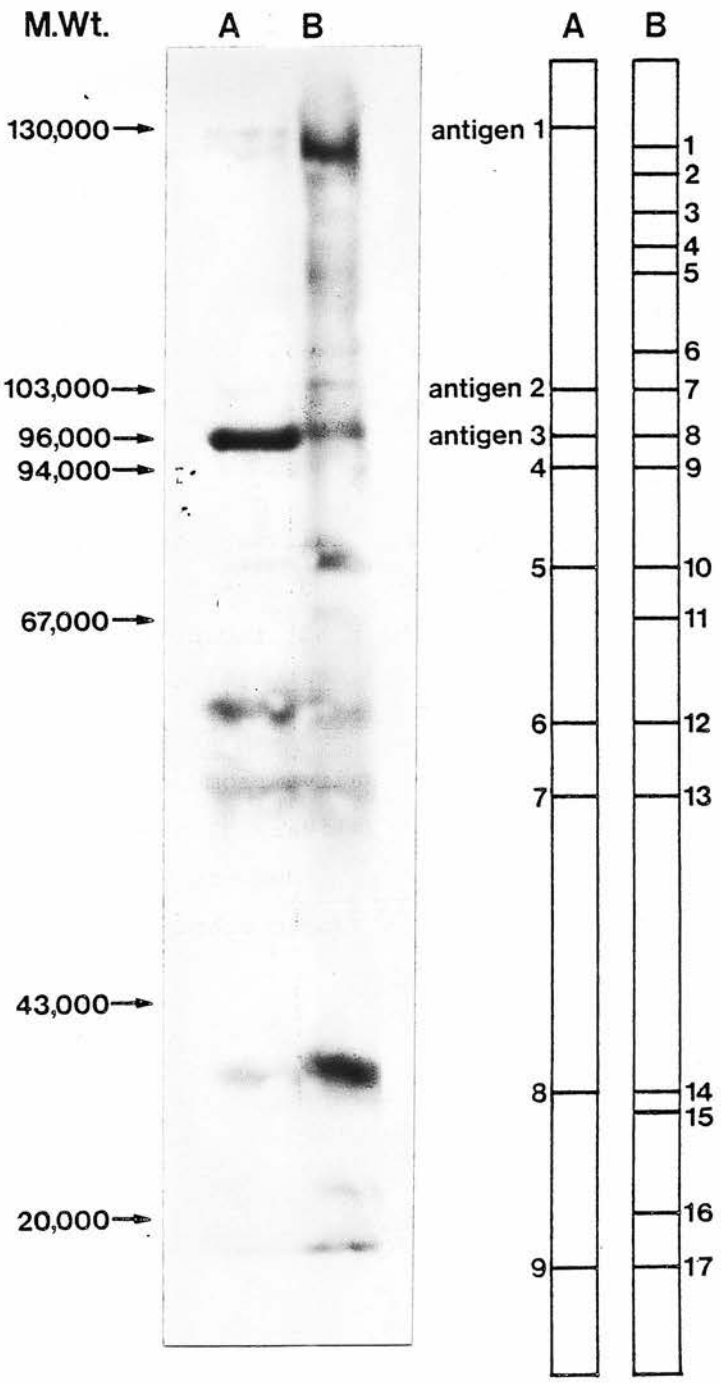

M.Wt.
A
B
130,000
103,000
96,000
94,000
67,000
43,000
20,000
A
B
antigen 1
antigen 2
antigen 3
4
5
6
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Figure 6.4 Proteins of saliva (Track A) and SGE (Track B) from 96 hour fed female H. a. anatolicum separated by SDS-PAGE on 7% acrylamide gel slabs; electroblotted and stained for glycoproteins using CON A - peroxidase (Rautenberg et al., 1980).

6.4

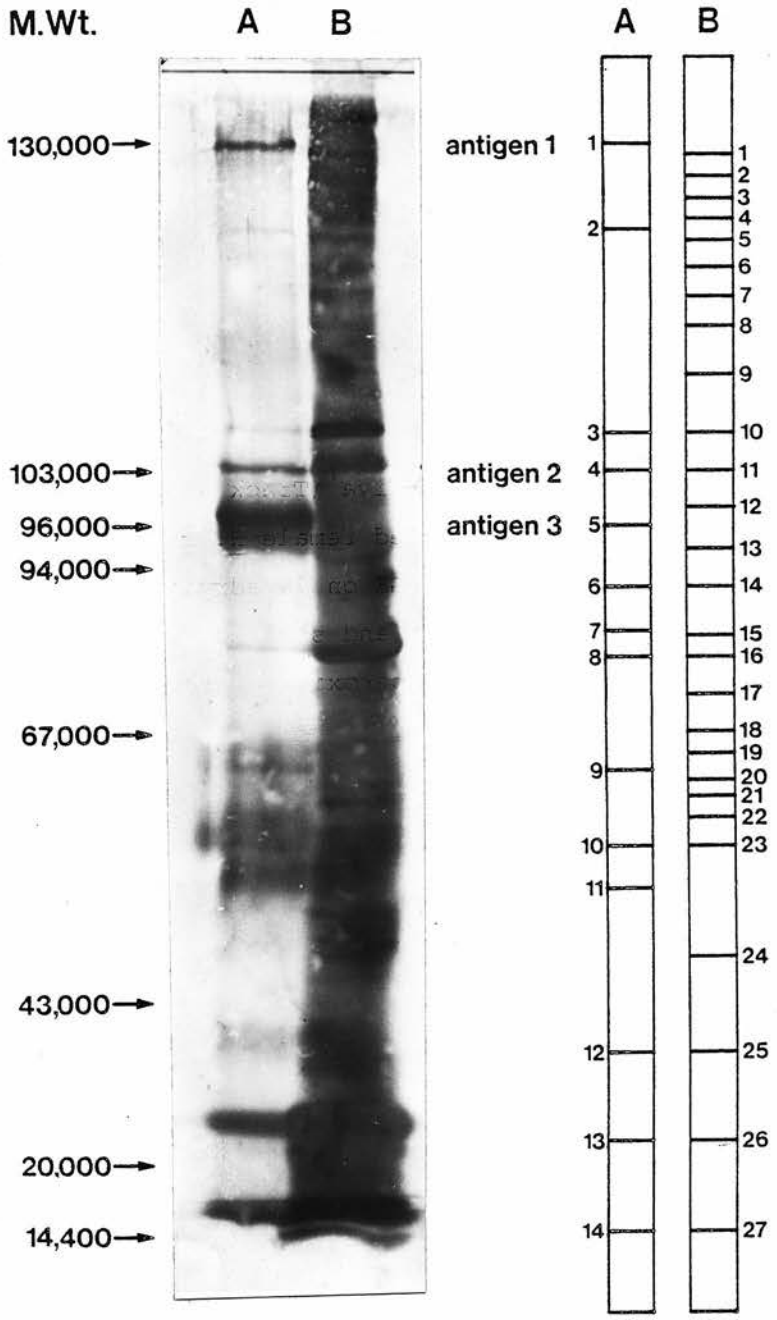

M.Wt.
A
B
130,000
103,000
96,000
94,000
67,000
43,000
20,000
14,400
antigen 1
antigen 2
antigen 3
A
B

Figure 6.5 Saliva from 96 hour fed female H. a. anatolicum ticks, separated by agarose isoelectric focusing (pH range 3 to 10). One half of the gel was stained for proteins using Coomassie blue (Track A) and the other half was stained for non-specific esterases (Track B).

pI = isoelectric point.

6.5

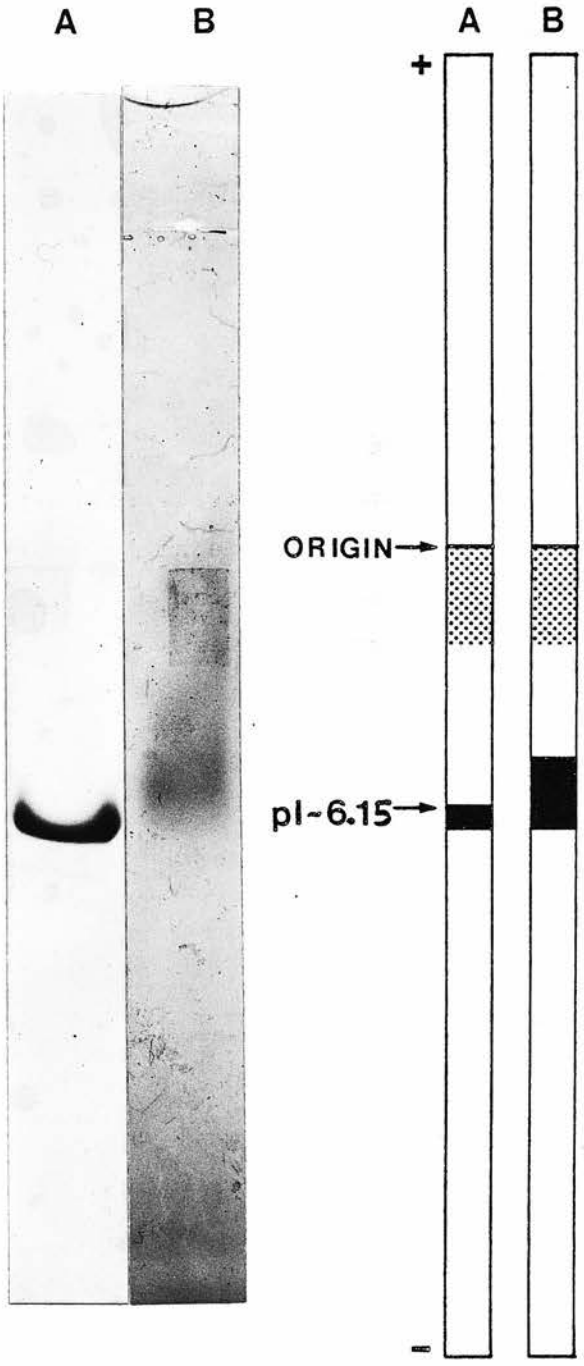


Figure 6.6 Proteins of saliva (Track A) and SGE (Track B) from 96 hour fed female H. a. anatolicum separated by 10% polyacrylamide gel polyacrylamide gel electrophoresis and stained for acid phosphatase.

6.6

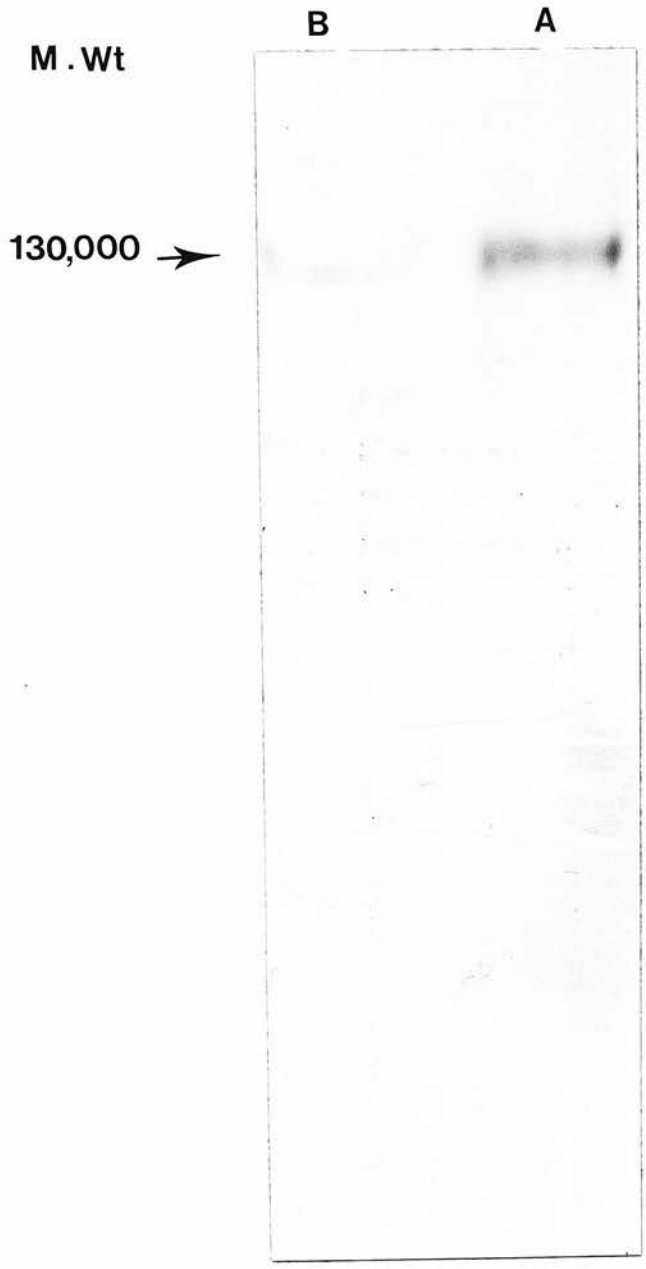
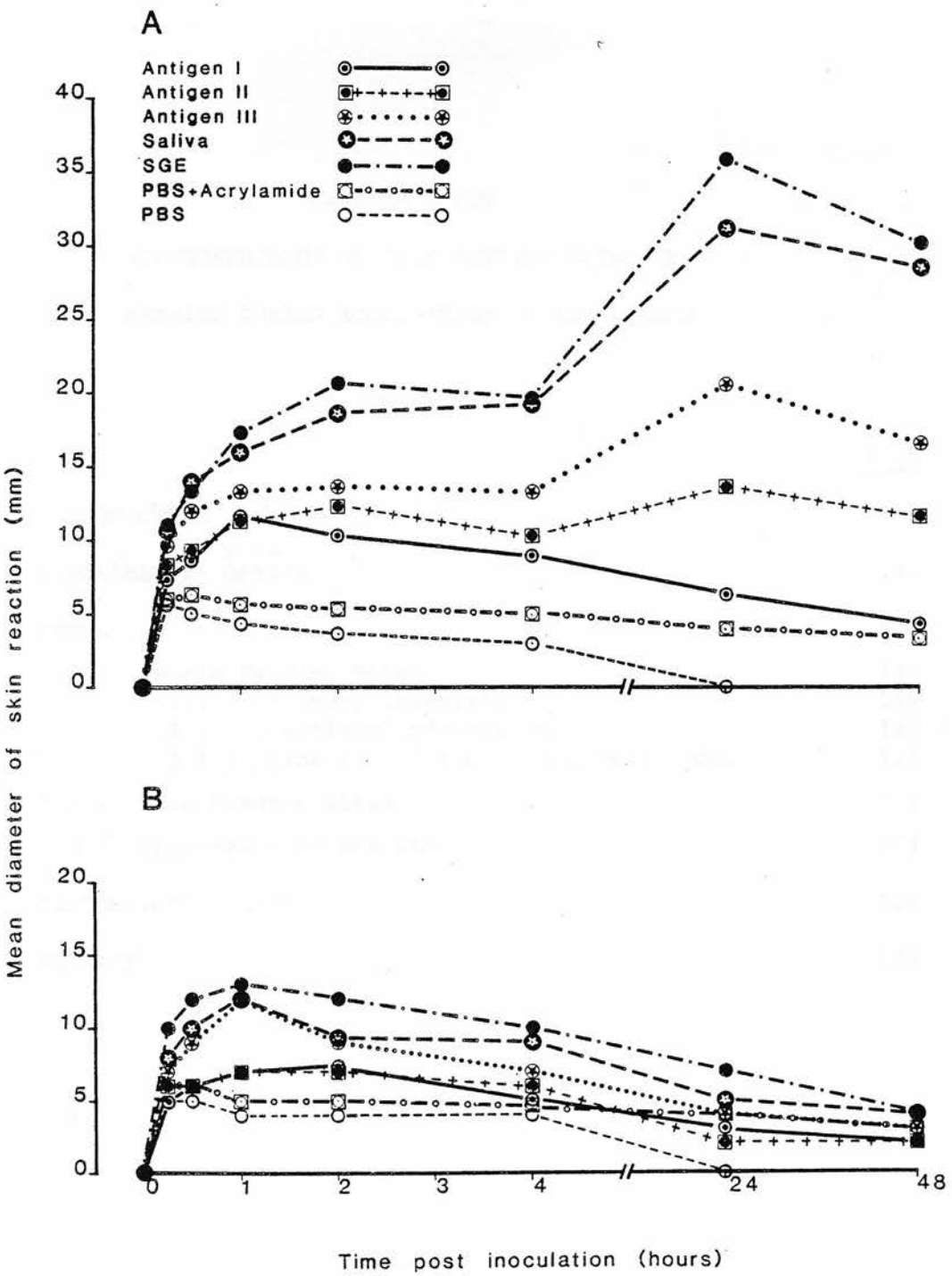


Figure 6.7 Skin reactivity of hypersensitized (A) and control (B) rabbits to intradermal inoculation of salivary antigens from 96 hour fed female H. a. anatolicum. Each point up to two hours represents a mean of four replicates and of three replicates from four to 48 hours.


A
Antigen I
Antigen II
Antigen III
Saliva
SGE
PBS+Acrylamide
PBS
40
35
30
25
20
15
10
5
0
B
20
15
10
5
0
0
1
2
3
4
24
48
Mean diameter of skin reaction (mm)
Time post inoculation (hours)

CHAPTER SEVEN

HISTOPATHOLOGY OF TICK FEEDING SITES IN RABBITS DURING ACQUISITION OF RESISTANCE

CONTENTS

7.1 INTRODUCTION

In recent years, the histology of tick feeding sites on naive and sensitized hosts has been investigated in a number of tick-host systems. The results of these studies have revealed that the host reaction to tick feeding is a complex phenomenon and depends greatly on the species of tick and host concerned, time post-attachment and whether or not the host is sensitized. However, the knowledge gained from these studies has greatly increased our understanding of immune effector mechanisms involved in the expression of resistance.

Tick feeding sites in resistant guinea-pigs exhibit large accumulations of basophils (Allen, 1973; Brown and Knap, 1981; Brown and Askenase, 1981; Krinsky *et al.*, 1982) and the effector role of these cells in the expression of resistance has been demonstrated by Brown *et al.* (1982a). The inability of hypersensitized rabbits to express significant resistance to *I. dammini* has been attributed to a weaker basophil response (Krinsky *et al.*, 1982).

The present investigation was undertaken with the following objectives:

(i) To define and compare the sequence of histological changes following primary and tertiary infestations by adult *H. a. anatolicum* ticks.

(ii) To gain an insight into cellular interactions inimical to ticks.

(iii) To monitor the expression of resistance following tertiary infestation.

7.2 EXPERIMENTAL DESIGN

Details of the tick infestation schedule are presented in Figure 7.1. Rabbits were subjected to infestation or reinfestation with 25 female and 25 male H. a. anatolicum ticks. After six hours, excess ticks were removed to leave 10 females and 10 males for histology, and 10 females and 15 males for monitoring the effect of resistance on tick feeding and egg laying. At each of 24, 72 and 144 hours after primary or tertiary infestation, three rabbits were killed. Four to six each of male and female tick feeding sites with attached mouthparts were removed from each rabbit. Feeding sites were fixed in Karnovsky's fixative, embedded in methacrylate and 1-2 μm thick sections were stained with acidic Giemsa's stain for quantification of histological responses.

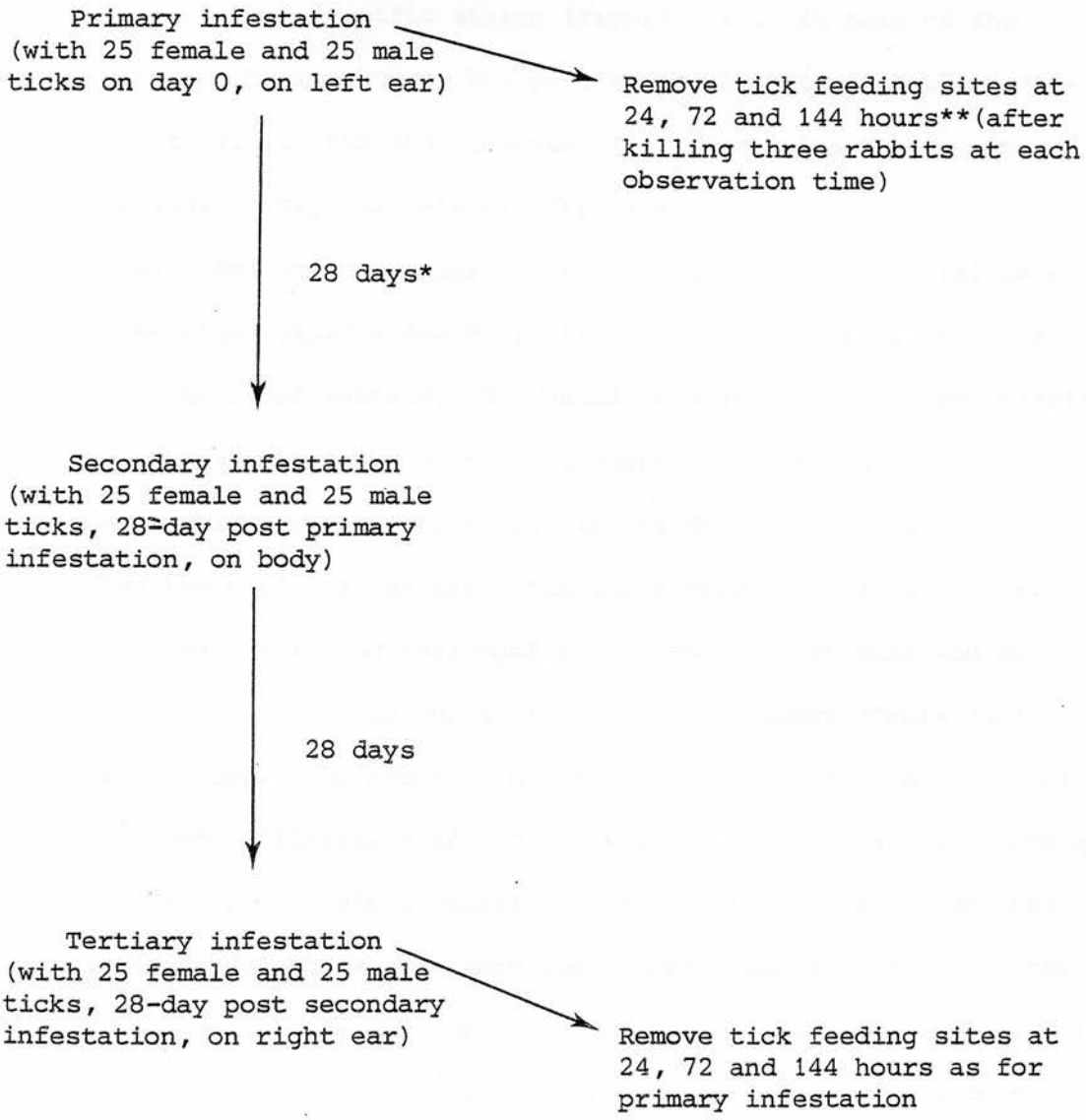
The acquisition of resistance was monitored by recording and comparing the time taken to engorgement, number of ticks engorged, engorged weight, weight of egg mass and failure to lay eggs, following primary and tertiary infestations.

7.3 RESULTS

7.3.1 Female Feeding Sites

7.3.1.1 Primary infestation: The mouthparts of H. a. anatolicum ticks were fully inserted into the dermis by 24 hours after attachment. They were ensheathed by the attachment cement along their entire length. Impressions of hypostomal digits were clearly seen in the cement cone when the mouthparts were accidentally peeled off during sectioning. The collagen bundles in contact with the cement cone had

Figure 7.1 Experimental design



*This infestation schedule was selected after consulting the relevant literature, so that these observations could be compared more closely with existing literature.

**This time sequence for quantification of histological responses was selected deliberately as it represented different stages of feeding.

lost their fibrillar appearance and were seen as a homogenous tissue mass. The homogenous character of collagen tissue was best appreciated with collagen specific stains (Figure 7.2). In most of the feeding sites the mouthparts had penetrated right through the cartilage (Figure 7.3). The chondriocytes in contact with the cement showed a loss of typical metachromatic staining.

Small foci of tissue destruction produced during initial penetration enlarged rapidly due to infiltration of inflammatory cells from adjacent blood vessels. Increased permeability of blood vessels in the immediate vicinity of the mouthparts resulted in oedema and many haemorrhagic foci (Figure 7.4) in the dermis. Neutrophils dominated the cellular reactions but there were also large numbers of mononuclear cells and eosinophils. Basophils were observed at most of the feeding sites but always in small numbers (Table 7.1).

At 72 hours, the reaction had changed very little in character. The increased infiltration of inflammatory cells resulted in increased size of the lesion. Blood vessels distant from the feeding mouthparts were engorged and there were many small haemorrhages. Oedema of the dermis had resulted in an overall thickening of the skin. Neutrophils exhibiting degeneration and necrosis were observed below the mouthparts and along the cement cone. Neutrophils were still the major component of the cellular infiltrate (Table 7.2) followed by mononuclear cells. Eosinophils and basophils were present in the same proportions as at 24 hours. However, there was a marked decrease in the number of mast-cells (Table 7.1).

Due to a progressive increase in the absolute numbers of inflammatory cells the lesion reached its maximum size at 144 hours and was

Table 7.1 Cellular responses in the dermis of rabbits at female H. a. anatolicum feeding sites

Infestation	Time after attachment (hours)	Numbers of cells/20 fields (Mean ± SE)				
		Mast-cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
Primary (n = 14)	24	8.4±1.3	13.7±3.4	56.5±13.7	342.0±66.2	127.6±31.2
Tertiary (n = 16)	24	2.8±0.5*	81.0±10.2*	387.0±41.7*	983.0±65.3*	419.2±33.4*
Primary (n = 14)	72	6.79±1.0	17.9±4.7	96.6±23.3	626.9±56.7	215.9±17.6
Tertiary (n = 17)	72	1.5±0.3*	102.1±13.4*	244.1±40.4*	538.8±43.9	405.9±18.4*
Primary (n = 13)	144	6.9±1.1	20.5±4.9	73.0±11.0	857.2±103.6	323.0±42.4
Tertiary (n = 17)	144	1.0±0.2*	107.0±12.6*	119.1±22.5	545.6±53.0*	458.5±31.7*

n = number of observations; *Primary vs Tertiary ($P < 0.01$);
Mononuclear cells included lymphocytes, monocytes and fibroblasts

Addendum at end

Table 7.2 Mean proportions of mast cells, basophils, eosinophils, neutrophils and mononuclear cells in the dermis of rabbits at female H. a. anatolicum feeding sites.

Infestation	Time after attachment (hours)	Mast cells %	Basophils %	Eosinophils %	Neutrophils %	Mononuclear cells %
Primary (n = 14)	24	3 (1-9)*	3 (0-17)	10 (4-14)	62 (41-75)	22 (13-33)
Tertiary (n = 16)	24	0 (0-1)	4 (2-9)	21 (11-41)	53 (35-66)	22 (14-32)
Primary (n = 14)	72	1 (0-2)	2 (0-4)	9 (2-22)	65 (52-75)	23 (13-31)
Tertiary (n = 16)	72	0 (0-1)	8 (2-17)	18 (7-37)	42 (31-54)	32 (23-40)
Primary (n = 13)	144	1 (0-2)	2 (0-4)	5 (2-10)	68 (60-76)	24 (18-32)
Tertiary (n = 17)	144	0 (0-<1)	9 (1-16)	9 (4-22)	44 (30-59)	38 (28-47)

n = number of observations
*Range of values
N.B. All the values are rounded up to the nearest whole number.

approximately four times larger than 24 hour lesions. Massive infiltration by neutrophils completely replaced the dermal connective tissue. The degeneration of neutrophils had resulted in further tissue necrosis. Extensive haemorrhage was indicated by the extravasation of a large number of erythrocytes throughout the dermis. Oedema of the dermis was obvious. The affected tissue stained weakly as compared to the healthy tissue. Hair follicles and sebaceous glands in the vicinity of the feeding lesion were unaffected. Localized perivascular cuffing by eosinophils in areas distant from the mouthparts was a common feature. Mast-cells, eosinophils and occasionally basophils exhibited slight degranulation. Neutrophils remained the predominant cell types throughout feeding. The proportion of eosinophils fell to half of that observed at 24 hours (Table 7.2).

7.3.1.2 Tertiary infestation: Following tertiary infestation the inflammatory response of the host was of much greater magnitude. All the histological changes observed following primary infestation were manifested more markedly. In addition, the sequence of cellular events had changed significantly.

At 24 hours, the lesion size was comparable to the 144 hour lesion following primary infestation (Figures 7.3 and 7.5). A majority of the tick feeding sites revealed the presence of intraepidermal vesicles away from the feeding mouthparts. These vesicles contained infiltrate rich in neutrophils (Figure 7.6). Eosinophils and mononuclear cells were rarely seen.

The cellular reactions in the dermis were characterized by massive degranulation of mast-cells and basophils especially along the

cement cone, at the dermo-epidermal junction and at the periphery of the lesion (Figure 7.7). Aggregation of eosinophils with a few intact basophils and mononuclear cells (Figure 7.8) at the periphery of the degranulation zone resulted in a distinct collar all around the feeding lesion. Eosinophils in the immediate vicinity of the mouthparts showed varying degrees of degranulation. Basophils were occasionally seen in the lumen of blood vessels and also in perivascular eosinophil cuffs. Oedema of the dermis and epidermis was significant. Blood vessels were dilated and packed with inflammatory cells.

The types and numbers of cells found at 24 hours after attachment has been summarised in Table 7.1. The neutrophils (53%) dominated the cellular infiltrate (Table 7.2). Eosinophils (21%) and mononuclear cells (22%) were the other significant cell types. Basophils were more common during tertiary infestation than during primary infestation.

As the feeding advanced there was an increase in the size of the lesion. At 72 hours, the epidermis showed spongiosis, oedema and hyperplasia. Vesiculation of the epidermis was more pronounced. In some cases the inflammatory exudate was seen seeping along the dermo-epidermal junction. Blood vessels were packed with leucocytes and were greatly dilated. There was hypertrophy and hyperplasia of endothelial cells and thickening of the basement membrane. Perivascular cuffing by eosinophils (Figure 7.9), a few mononuclear cells and basophils was a regular feature. There was extensive necrosis of the dermis. Degranulation of basophils and eosinophils was of a much

greater degree and mast-cells occurred rarely. Fibroblasts at the periphery of the lesion appeared enlarged.

By 144 hours, the lesions had reached their largest size (Figure 7.10). The oedema and necrosis of the dermis was greater in intensity. Analysis of the cellular infiltrate showed that the eosinophils (9%) had decreased in proportion, whereas basophils (9%) had increased both in absolute numbers and in proportions (Tables 7.1 and 7.2; Figure 7.11). The proportion of mononuclear cells had increased markedly forming 38% of the cellular infiltrate.

7.3.1.3 Kinetics of individual cell types in tissues:

(i) Mast-cells - The number of mast-cells observed in the skin of uninfected control rabbits was fairly small. The changes observed in the mean number of mast-cells are presented in Figure 7.12. There was a decrease in the mean number of mast-cells due to degranulation following primary and tertiary infestation (Table 7.1). However, degranulation following tertiary infestation was of much greater magnitude (Figure 7.7) and mast-cells were rarely seen from 24 hours post-attachment onwards.

(ii) Basophils - Basophil infiltration at tick feeding sites was noticed as early as 24 hours after attachment and showed a gradual increase as the feeding progressed (Figure 7.12). Basophil numbers reached a maximum at 144 hours, and they comprised 2% of the cellular infiltrate.

There was a dramatic increase in the number of basophils following tertiary infestation. In spite of massive degranulation the mean number of basophils at 24 hours after tertiary infestation was four

times that observed during primary infestation (Table 7.1). Peak tissue basophilia was observed at 144 hours after tertiary infestation, when they formed 9% of the cellular infiltrate (Table 7.2).

(iii) Eosinophils - Eosinophils were rarely seen in the skin of control rabbits. However, they infiltrated tick feeding sites in significant numbers as early as 24 hours after attachment in primary infestations. They formed 10% of the infiltrate at this time. Their mean numbers increased till 72 hours and then decreased gradually, forming only 5% of the inflammatory cells at 144 hours (Table 7.2).

Following tertiary infestation the mean number of eosinophils reached a maximum (21%) at 24 hours (Table 7.2) and they were the third most abundant cell type. However, their proportion fell significantly towards the end of feeding. At 144 hours they constituted only 9% of the cellular infiltrate.

(iv) Neutrophils - Neutrophils were the most abundant cell type at all feeding times studied. They formed 62% to 68% of the inflammatory infiltrate following primary, and 42% to 53% following tertiary infestations (Table 7.2). Tissue destruction at the tick feeding site paralleled the frequency of neutrophil infiltration and degeneration.

(v) Mononuclear cells - These were the second most predominant cell type throughout primary and tertiary infestation (Table 7.2). There was a progressive increase in their mean number (Figure 7.12) reaching a maximum (24%) at 144 hours after attachment.

Following tertiary infestation their number peaked at 144 hours, at which time they comprised 38% of the cellular infiltrate (Table 7.2).

7.3.2 Male Feeding Sites

The lesions produced by feeding males were far smaller than those of females. However, the general histological changes, nature and sequence of cellular events at male feeding sites following primary and tertiary infestations were the same as those seen with female ticks. Therefore, the histological changes will not be re-described.

Quantitative histological analysis of the cellular infiltrates has been presented in Tables 7.3, 7.4 and Figure 7.13. Statistically, the only differences observed as compared to female feeding sites at 5% level of significance were: (i) a smaller degree of mast-cell degranulation observed at 24 hours after primary and 144 hours after tertiary attachments; (ii) comparatively low infiltration of eosinophils 24 hours after tertiary infestation and (iii) weaker neutrophil response at 72 hours after primary infestation.

7.3.3 Expression of Resistance

The effect of resistance on tick feeding and egg laying has been summarised in Table 7.5. Resistance was manifested by prolongation of feeding time, reduction in egg production, failure to lay eggs and sometimes death *in situ* (Table 7.5). The mean time to engorgement increased from 7.8 days following primary infestation to 10.6 days following tertiary infestation. In addition, the engorged weights fell dramatically by 69% following tertiary infestation. The distribution of engorged weights following primary and tertiary infestations has been presented in Figure 7.14.

Table 7.3 Cellular responses in the dermis of rabbits at male H. a. anatolicum feeding sites

Infestation	Time after attachment (hours)	Number of cells/20 fields (Mean ± SE)				
		Mast-cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
Primary (n = 17)	24	11.1±1.2	14.2±3.4	60.8±14.5	322.3±66.4	109.2±22.6
Tertiary (n = 15)	24	3.2±0.6*	60.3±8.0*	241.9±31.2*	918.9±85.6*	470.5±43.0*
Primary (n = 15)	72	6.9±1.0	16.5±4.0	90.3±22.5	327.3±52.8	169.5±29.3
Tertiary (n = 15)	72	2.1 ±0.4*	80.8±10.8*	281.8±51.1*	776.4±126.7*	409.4±24.6*
Primary (n = 15)	144	6.9±0.8	23.0±5.3	90.3±10.3	828.7±89.5	313.1±32.6
Tertiary (n = 16)	144	1.9±0.2*	105.1±7.0*	116.1±19.2	705.1±75.4	443.2±33.7*

n = number of observations; *Primary vs. Tertiary ($P < 0.01$);
Mononuclear cells included lymphocytes, monocytes and fibroblasts.

Addendum at end

Table 7.4 Mean proportions of mast cells, basophils, eosinophils, neutrophils and mononuclear cells in the dermis of rabbits at male H. a. anatolicum feeding sites.

Infestation	Time after attachment (hours)	Mast-cells %	Basophils %	Eosinophils %	Neutrophils %	Mononuclear cells %
Primary (n = 17)	24	5 (1-25)*	4 (0-18)	14 (4-40)	56 (14-75)	21 (16-29)
Tertiary (n = 15)	24	0.19 (0-1)	4 (1-6)	15 (5-28)	53 (42-64)	28 (17-34)
Primary (n = 15)	72	2 (1-5)	2 (0-4)	13 (4-24)	55 (41-69)	28 (14-37)
Tertiary (n = 15)	72	0 (0-<1)	7 (2-12)	17 (6-31)	47 (29-70)	29 (16-43)
Primary (n = 15)	144	1 (0-3)	2 (0-4)	8 (2-20)	64 (54-82)	25 (14-34)
Tertiary (n = 16)	144	0 (0-1)	8 (5-14)	8 (2-15)	50 (21-61)	34 (23-59)

n = number of observations
*Range of values
N.B. All values are rounded up to the nearest whole number.

Table 7.5 Effect of resistance on feeding and egg laying in female H. a. anatolicum

	Primary infestation	Tertiary infestation
Mean time to engorgement (days)	7.8±0.1* (n = 60)	10.6±0.3 (n = 28)**
Mean weight of engorged females (mg)	353.0±13.1 (n = 60)	109.9±9.1 (n = 28)**
Mean weight of egg mass (mg)	191.9±9.2 (n = 59)	40.7±4.2 (n = 24)**
Percent eggs laid of engorged weight (mg)	53.3	33.8
Failure to lay eggs (%)	-	14.3
Death in situ (%)	-	6.7

n = number of observations; *Mean ± Standard error; **Primary vs. Tertiary ($P < 0.01$)

There was a significant reduction in the proportion of eggs laid to fed weight on tertiary infestation (Table 7.5). All the ticks fed on naive hosts laid viable eggs, whereas 14% of the females failed to lay eggs following tertiary infestation. However, there was no effect on egg hatching.

7.4 DISCUSSION

The present study indicates that the cellular infiltrate changes in character and magnitude during feeding and differs significantly from the primary infestation to subsequent infestations. The host responses following infestation by adult H. a. anatolicum were typical of an inflammatory reaction with vascular dilation, dermal oedema, haemorrhage and infiltration of leucocytes into the tick feeding sites. Inflammation is by its nature a beneficial response in an evolutionary sense, to effectively deal with infection or physical tissue damage (Henson, 1982).

However, in addition to mechanical damage, the tissue destruction and increased permeability observed as early as 24 hours after attachment could also be due to the cytolytic effect of weakly hydrolytic enzymes (Tatchell, 1971; Schleger et al., 1976; 5.3.2) and inflammatory agents (Dickinson et al., 1976; Higgs et al., 1976; Shemesh et al., 1979) present in the saliva of ixodid ticks. The homogenization of collagen bundles in contact with the cement cone might be due to the effect of aminopeptidase present in the cement cone of H. a. anatolicum (5.3.2.3).

Sequential, quantitative analysis of the cellular infiltrate showed that neutrophils were the dominant cell type throughout primary (62-68%) and tertiary (42-53%) infestations. Massive infiltration of neutrophils following primary infestation could be due to the generation of chemotactic factors for neutrophils by salivary secretions of ticks (Berenberg et al., 1972). However, a marked neutrophil response following tertiary infestation, could well be due to the formation of immune complexes (Cochrane, 1971) at tick feeding sites in addition to the effect of salivary secretions. The extent of collagen destruction at tick feeding sites coincided with the degree of neutrophil infiltration and degeneration. This is in agreement with the findings of Tatchell and Moorhouse (1970), and Theis and Budwiser (1974) who demonstrated that tissue destruction following R. sanguineus feeding on dogs was neutrophil dependent. The preponderance of neutrophils, massive tissue destruction, increased vascular permeability, oedema and haemorrhage following tertiary infestation suggested the involvement of an Arthus type of reaction (Henson, 1982).

The varying degree of mast-cell degranulation following primary infestation could be due to degranulating agents in saliva (Geczy et al., 1971), accidental disruption of cells, or activation of complement (Movat, 1971). Agents capable of causing mast-cell degranulation have been demonstrated in insect saliva (Johnson and Erdos, 1973) and venom (Habermann, 1972). Similar observations on mast-cell degranulation following primary tick infestation have been made by Tatchell and Moorhouse (1968), Theis and Budwiser (1974) and Brossard and Fivaz (1982).

The greater degree of mast-cell degranulation on tertiary infestation could be the result of interactions between tick salivary antigens and mast-cell bound homocytotropic and/or IgG antibodies (Leid, 1982). Development of homocytotropic (Riek, 1962; Willadsen et al., 1978; McGowan et al., 1979) and IgG (Brossard, 1977; Fujisaki, 1978; Brown et al., 1982b) antibodies has been demonstrated in a number of tick-host systems. The development of resistance to H. leporispalustris (McGowan et al., 1982) was correlated with the development of homocytotropic antibodies. Degranulation of mast-cells resulted in release of vasoactive amines and chemotactic factors for eosinophils (ECF-A), basophils and neutrophils (NCF).

A significant infiltration of eosinophils following primary tick infestation could be due to intrinsic chemotactic activity of tick saliva (Allen et al., 1977). Tanaka and Torisu (1978) reported the release of eosinophil chemotactic factors directly from helminthic parasites during tissue invasion.

The marked increase in eosinophil numbers and proportions at 24 hours after tertiary infestation coincided with the findings of Schleger et al. (1976), Allen et al. (1977), Brown and Knap (1981), Brossard and Fivaz (1982) and Brown et al. (1983). This could be due to the chemotactic factors released by degranulated mast-cells, antigen-antibody interaction (Kay and Austen, 1972), complement activation (Kay, 1970) or lymphocyte mediated responses (Colley, 1973).

The dramatic decrease in the number of eosinophils at 72 hours and thereafter (Table 7.1) following tertiary infestation could be due to their degranulation to modulate the effect of mast-cell derived

mediators (Weller and Goetzl, 1979). Eosinophils have been found to be important mediators of resistance against ticks (Brown et al., 1982a). It is possible that the eosinophil components might damage the gut epithelia of ticks or disturb other vital physiological functions. However, the treatment of tick resistant hosts with specific anti-eosinophil major basic protein (MBP) serum failed to block the expression of resistance (Brown and Askenase, 1983); but the role of other proteins or enzymes in effecting resistance to ticks is obscure.

In contrast to the tick-guinea-pig system (Allen, 1973; Bagnall and Doube, 1975; Brown and Askenase, 1981; Brown and Knap, 1981; Brown et al., 1984), basophils formed significantly small proportions of the cellular infiltrate (4-9%) on H. a. anatolicum challenge infestation in rabbits. However, this is in agreement with the findings of Brossard and Fivaz (1982) on I. ricinus feeding sites in rabbits. The weaker basophil response in sensitized rabbits as compared to guinea-pigs could be because of the low mast-cell density of rabbit skin (Riley, 1959). Unlike the tick-guinea-pig system, the early (24 hours after attachment) infiltration of basophils at tick feeding sites on rabbits was intriguing. However, this could be a response to early mast-cell degranulation observed following primary infestation.

The increased influx of basophils on tertiary infestation could have been induced by factors from degranulated mast-cells, sensitized T lymphocytes (Wikel and Allen, 1976a) and by antibody IgG (Brown et al., 1982b) as well as complement (Wikel, 1979) dependent mechanisms. On tertiary infestation the massive degranulation of basophils

(Figure 7.7) could have been the result of interaction between salivary antigen and specific homocytotropic antibody bound to the surface of basophils. Using a degranulation test Brossard et al. (1982) demonstrated progressive sensitization of circulating basophils to tick salivary antigens.

In the guinea-pig tick system the rejection of larval ticks coincided with the infiltration and degranulation of basophils (Allen, 1973; Bagnall and Doube, 1975; Brown and Askenase, 1981; Brown et al., 1983). The expression of resistance was completely blocked by anti-basophil serum (Brown et al., 1982a), which further suggested their important effector role in resistance to ticks.

Basophils and mast-cell granules are a rich source of histamine (Askenase, 1977), while the eosinophils modulate the effect of histamine (Gleich et al., 1979) and are also postulated to translocate histamine to the tick attachment site (Schleger et al., 1981). Thus the net consequence of mast-cell-basophil-eosinophil interaction is increased levels of histamine at tick feeding sites. Histamine has been shown to have a direct effect on tick attachment and feeding (Kemp and Bourne, 1980; Paine et al., 1983). Resistance to B. microplus (Tatchell and Bennet, 1969) and D. andersoni (Wikel, 1982) was significantly suppressed with antihistamine treatment of resistant hosts. But how histamine affects resistance is not clear.

In an in vitro feeding system the addition of histamine and 5-hydroxytryptamine resulted in a significant reduction in recordings associated with sucking and salivation (Paine et al., 1983). Isolated cockroach salivary glands failed to register any secretory activity

on stimulation with histamine and secretory responses elicited by 5-hydroxytryptamine were feeble as compared to catecholamines (House and Ginsborg, 1979). In the present study the salivary glands of ticks fed on resistant hosts exhibited a massive accumulation of secretory granules indicating impaired secretion. Therefore, from this evidence it is reasonable to hypothesize that the histamine or other pharmacological mediators released during hypersensitivity reactions affects catecholaminergic transmission at the neuroglandular junctions. It is possible that histamine acts as an antagonist to catecholamines. On the other hand if histamine is an agonist to catecholamines it may be very weak and may occupy the catecholamenergic receptors in such a way that it competes with catecholamines and suppresses salivary secretions.

The significant decrease in the weight of fed females, increase in time to engorgement, poor egg laying and failure to lay eggs following tertiary infestation might have been the result of poor nutrition. The poor nutrition could have resulted from inactivation of salivary enzymes by host antibodies at the feeding site or in the gut of the tick. However, it could also be the result of poor salivary secretions effected by histamine or other enzymes of basophils and eosinophils.

The death of ticks *in situ* on resistant hosts suggested a toxic effect of mediators released during cellular interactions on vital physiological centres. Similar effects on tick feeding and egg production have been reported by Branagan (1974) and Bowessidjaou *et al.* (1977).

7.5 SUMMARY

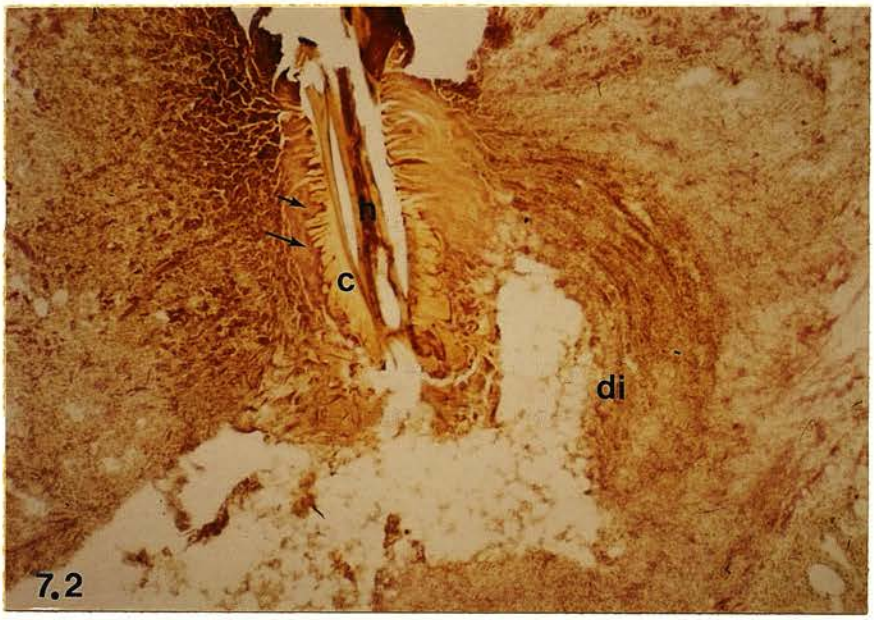
The histological picture of tick feeding sites on rabbits showed that the vascular damage was caused by the tick whilst tissue damage might have been caused by the host responses induced by salivary secretions. Quantification of the cellular response indicated that neutrophils (62-68%) were the dominant cells throughout feeding, followed by mononuclear cells (22-24%) and eosinophils (5-10%). Basophils were seen in most of the tick feeding sites but always in small numbers. On tertiary infestation the lesions were characterized by massive degranulation of mast-cells and basophils. Localized eosinophil aggregation especially at the periphery of the feeding lesion was a constant feature at 24 hours post attachment. As the feeding advanced there was an increase in the proportions of infiltrating mononuclear cells from 22 to 38%, of basophils from 4 to 9% and a decrease in the proportion of eosinophils from 21 to 9%. Neutrophils (42-53%) remained the dominant cells throughout tertiary feeding. There was no significant difference in the proportion of infiltrating cells in the male and female tick feeding sites, although the lesions produced by female feedings were of much greater size than those of males. The pharmacological mediators released by degranulating mast-cells, basophils and eosinophils appeared to be the main cause of reduced feeding by ticks on tertiary infestation.

Possible mechanisms by which these mediators effect resistance are suggested and discussed.

Figure 7.2 Primary infestation, 144 hours after attachment of female H. a. anatolicum to the ear of a rabbit. Note the homogenous character of collagen bundles (arrows). Cryostat section (Van Gieson Technique, x 44).

Figure 7.3 Primary infestation, 144 hours after attachment of female H. a. anatolicum to the ear of a rabbit. Note the cartilage pierced by the mouth-parts of the tick. Methacrylate section (Giemsa, x 44).

c = cement cone; ct = cartilage; di = dermal infiltrate; ep = epidermis; h = hypostome.



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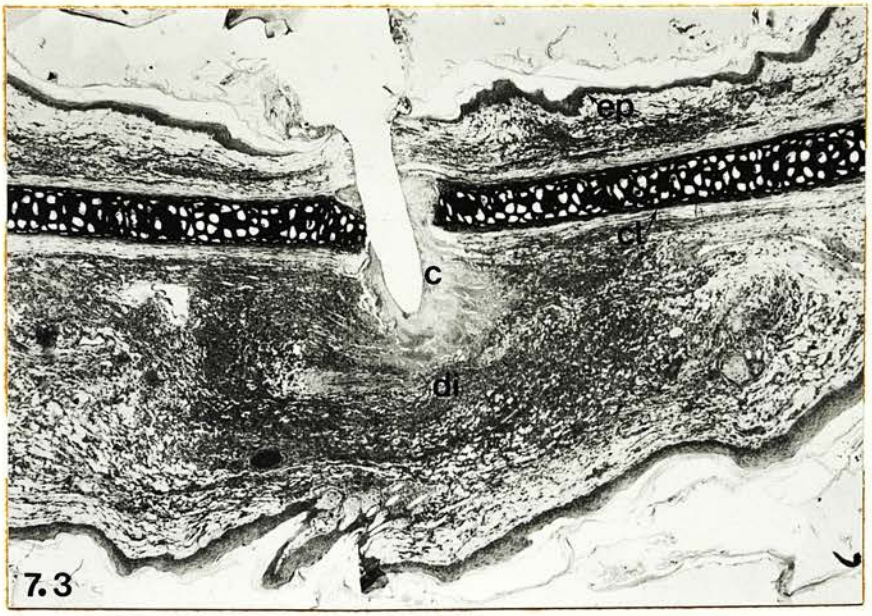
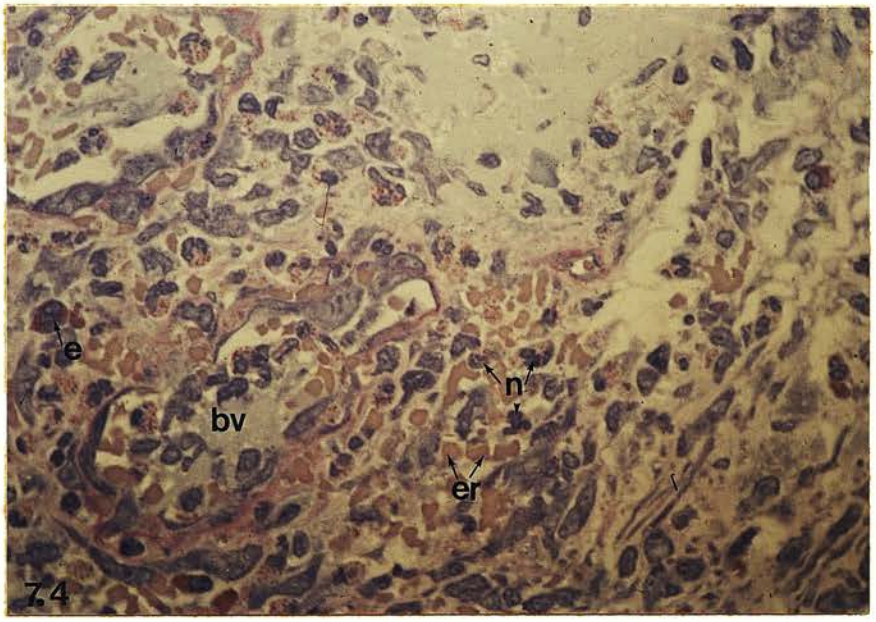


7.3

Figure 7.4 Primary infestation, 24 hours after attachment of female H. a. anatolicum to the ear of a rabbit. The dermis directly beneath the tick attachment site. Note the increased vascular permeability of blood vessels. Methacrylate section (Giemsa, x 544).

Figure 7.5 Tertiary infestation, 24 hours after attachment of female H. a. anatolicum to the ear of a rabbit. Note the cellular infiltrate is more marked than in the primary infestation (Figure 7.3). Methacrylate section (Giemsa, x 44).

bv = blood vessels; c = cement cone; ct = cartilage;
di = dermal infiltrate; e = eosinophil; ep = epidermis;
er = erythrocytes; n = neutrophils.



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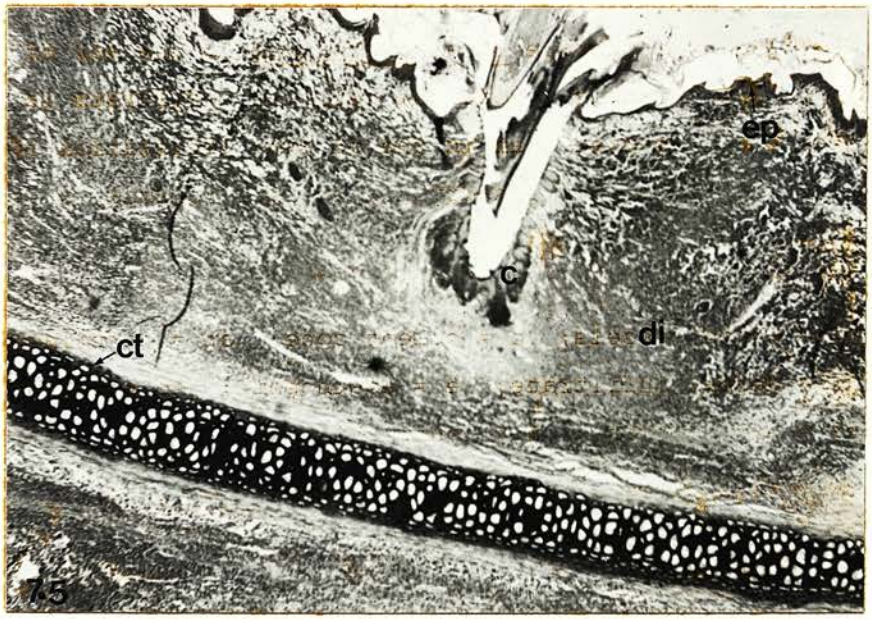
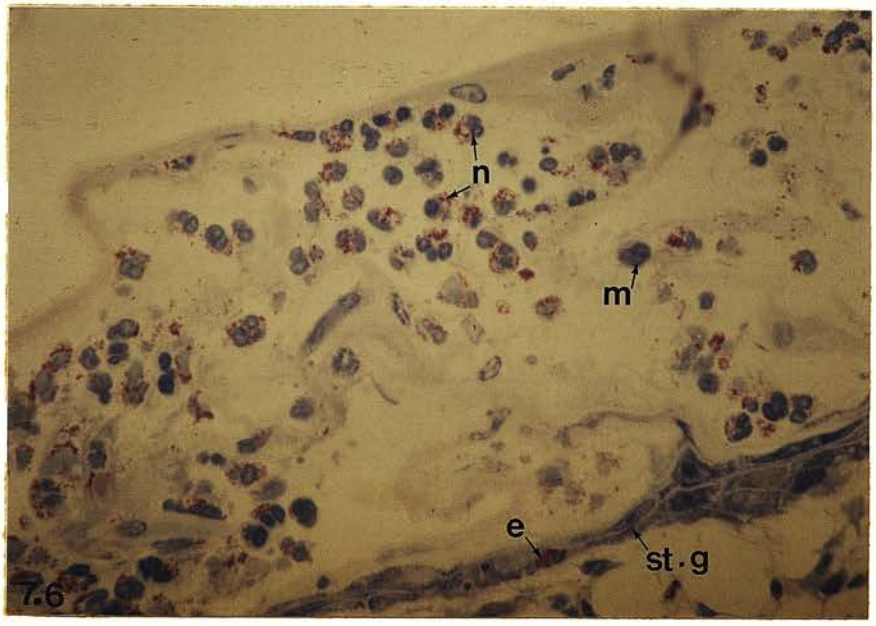


7.5

Figure 7.6 Tertiary infestation, 24 hours after attachment of female H. a. anatolicum to the ear of a rabbit. An epidermal vesicle at a distance from the mouthparts of the tick. Methacrylate section (Giemsa, x 544)

Figure 7.7 Tertiary infestation, 24 hours after attachment of female H. a. anatolicum to the ear of a rabbit. Massive degranulation of basophils and mast cells at the periphery of the feeding lesion. Note the numerous free metachromatic granules (at arrows). Methacrylate section (Giemsa, x 544).

b = basophils; e = eosinophils; m = mononuclear cells; mc = mast-cell; n = neutrophils; st.g = stratum germinativum.


n
m
e
st.g
7.6

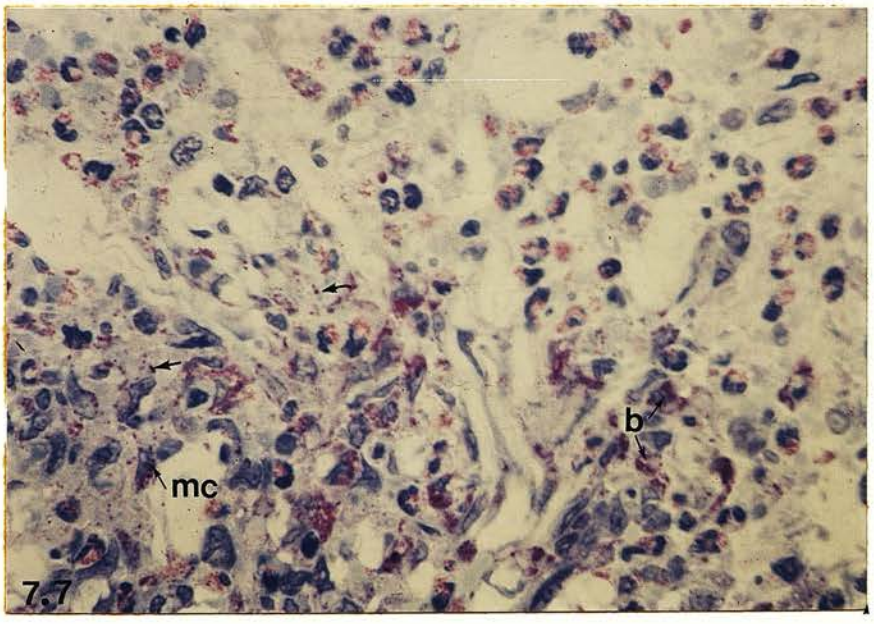
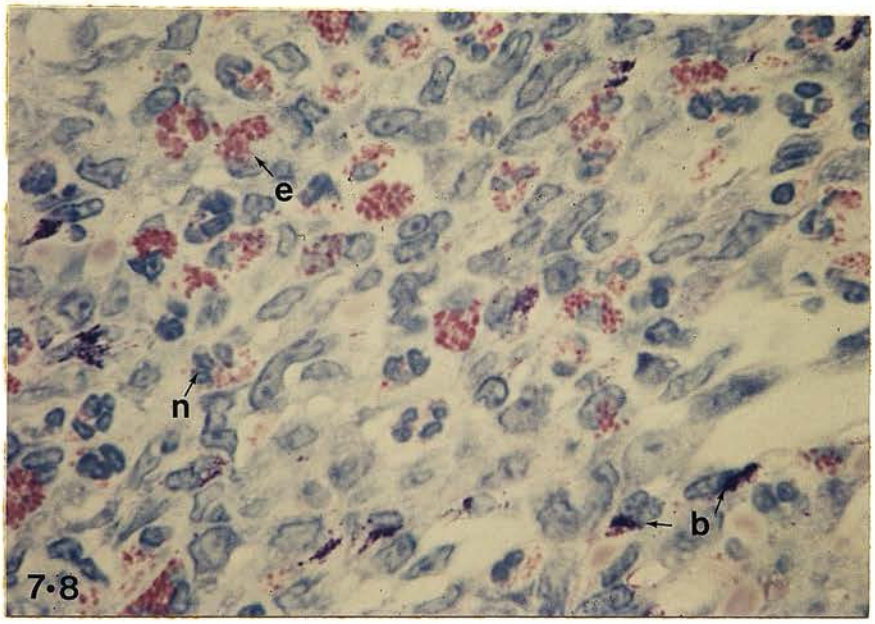

b
mc
7.7

Figure 7.8 Tertiary infestation, 24 hours after attachment of female H. a. anatolicum to the ear of a rabbit. The dermal infiltrate at the periphery of the mast-cell/basophil degranulation zone. Methacrylate section (Giemsa, x 1,088).

Figure 7.9 Tertiary infestation, 72 hours after attachment of female H. a. anatolicum to the ear of a rabbit. The dermis at a distance from the mouthparts of the ticks. Methacrylate section (Giemsa, x 544).

b = basophils; bv = blood vessel; e = eosinophils;
m = mononuclear cells; n = neutrophils.



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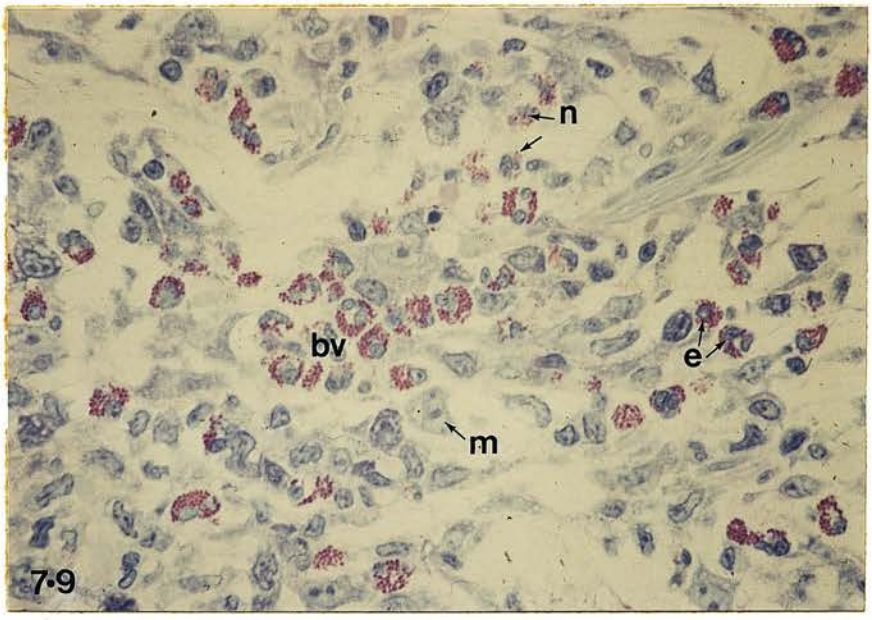


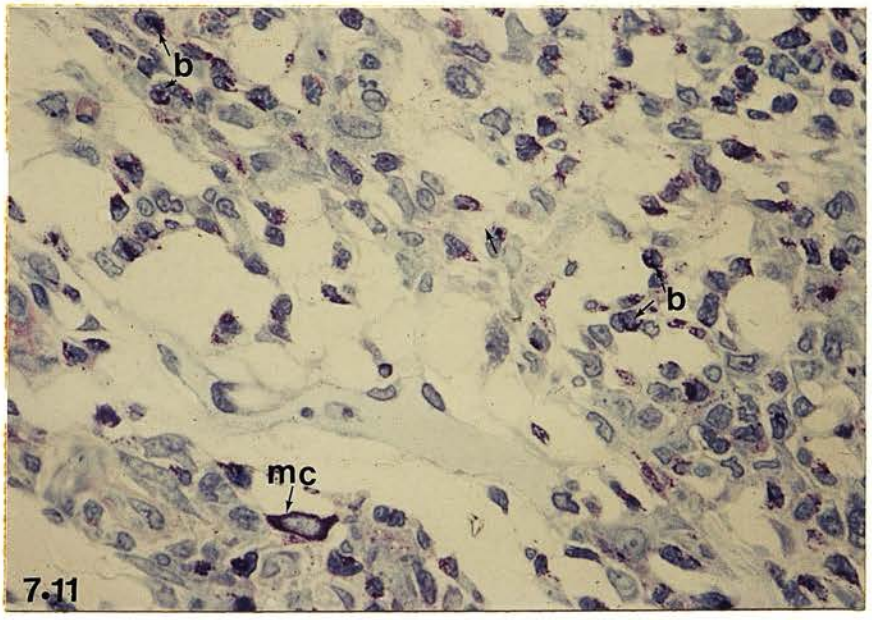
7·9

Figure 7.10 Tertiary infestation, 144 hours after attachment of female H. a. anatolicum to the ear of a rabbit. The maximal size of the lesion. Note the marked oedema and inflammatory infiltrate in the dermis. Methacrylate section (Giemsa, x 44).

Figure 7.11 Tertiary infestation, 144 hours after attachment of female H. a. anatolicum to the ear of a rabbit. The dermis at a distance from the mouthparts showing an overwhelming infiltration of basophils. Note the morphologic features distinguishing a fixed tissue mast-cell from basophils. Methacrylate section (Giemsa, x 544).

b = basophils; c = cement cone; di = dermal infiltrate; ep = epidermis; ep.v = epidermal vesicle; mc = mast-cell.

7.10



7.11

Figure 7.12 Mean cellular responses in the dermis of rabbits at female H. a. anatolicum feeding sites.

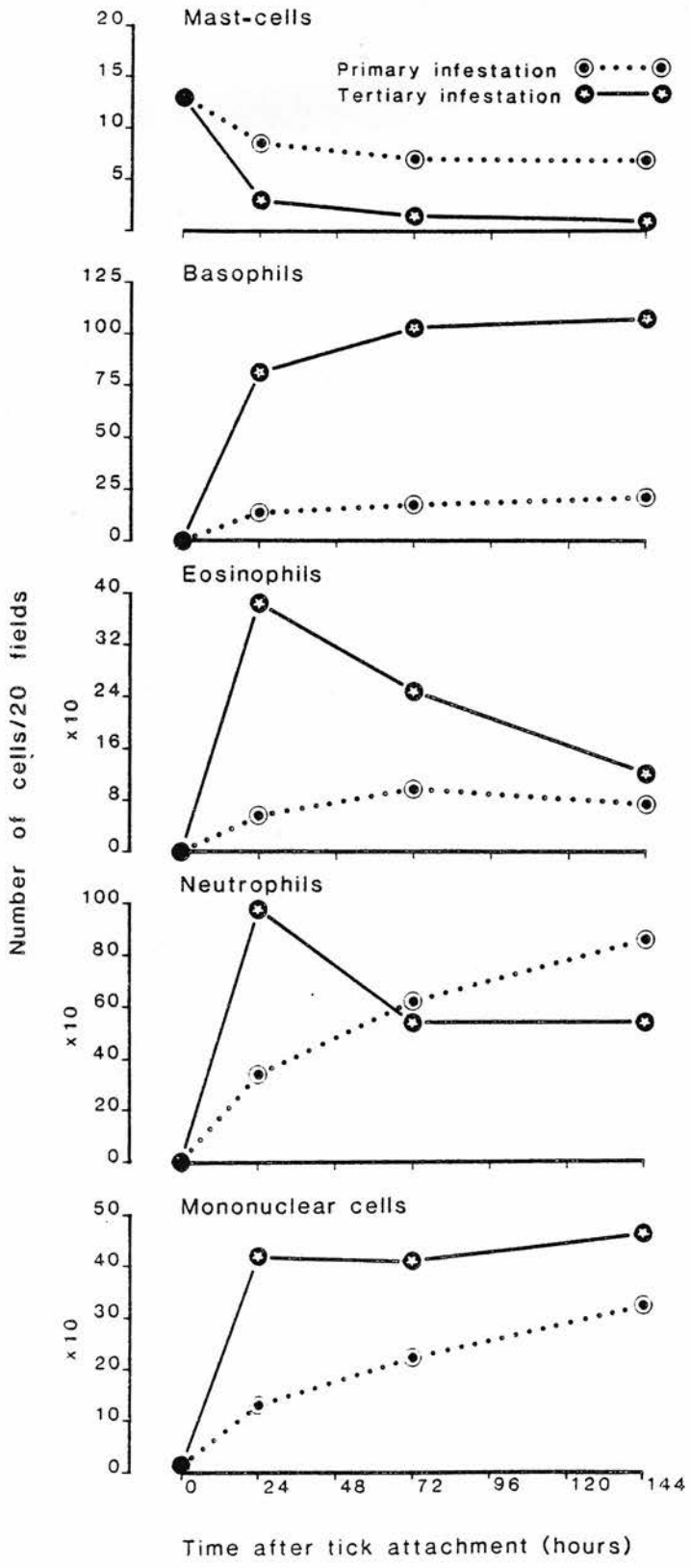

Mast-cells
Primary infestation
Tertiary infestation
20
15
10
5
Basophils
125
100
75
50
25
0
Eosinophils
40
32
24
16
8
0
x10
Neutrophils
100
80
60
40
20
0
x10
Mononuclear cells
50
40
30
20
10
0
x10
Number of cells/20 fields
0
24
48
72
96
120
144
Time after tick attachment (hours)

Figure 7.13 Mean cellular responses in the dermis of rabbits at male H. a. anatolicum feeding sites.

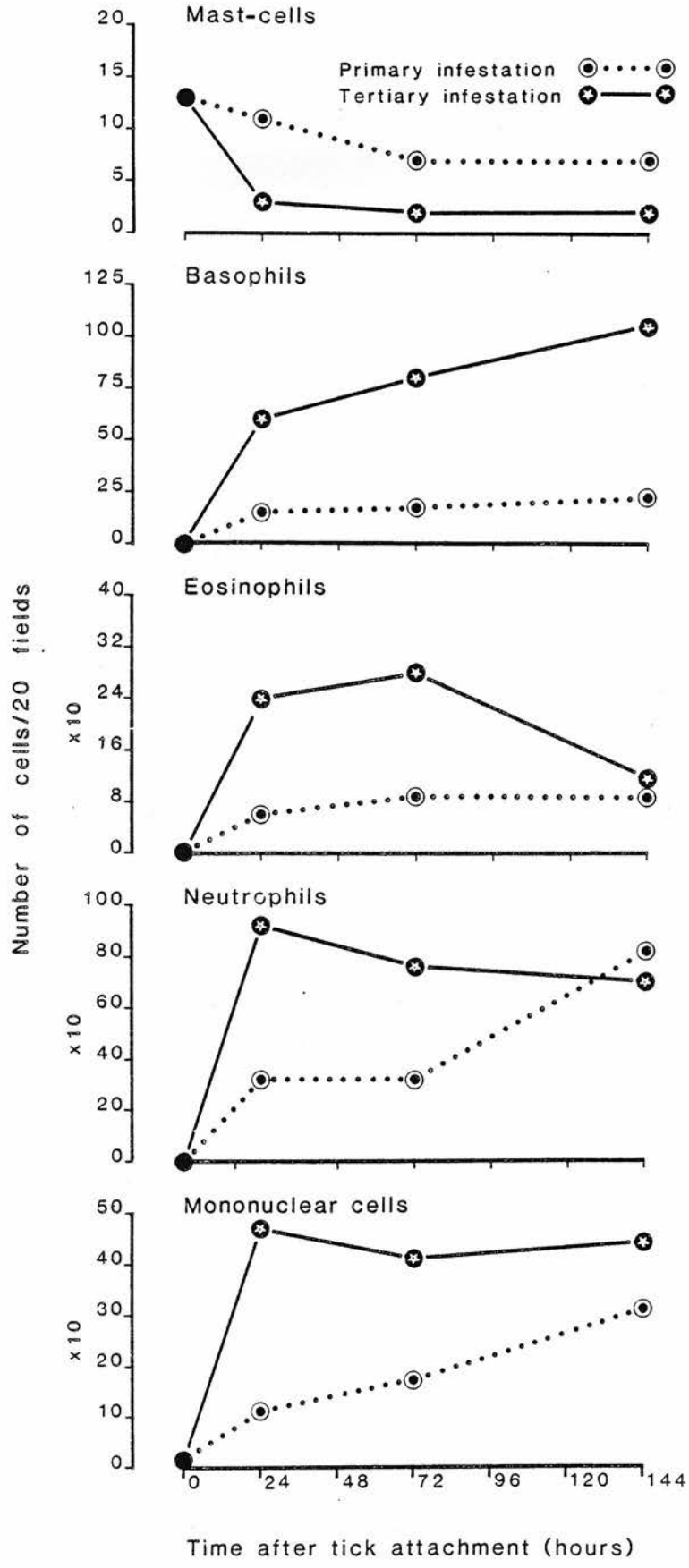
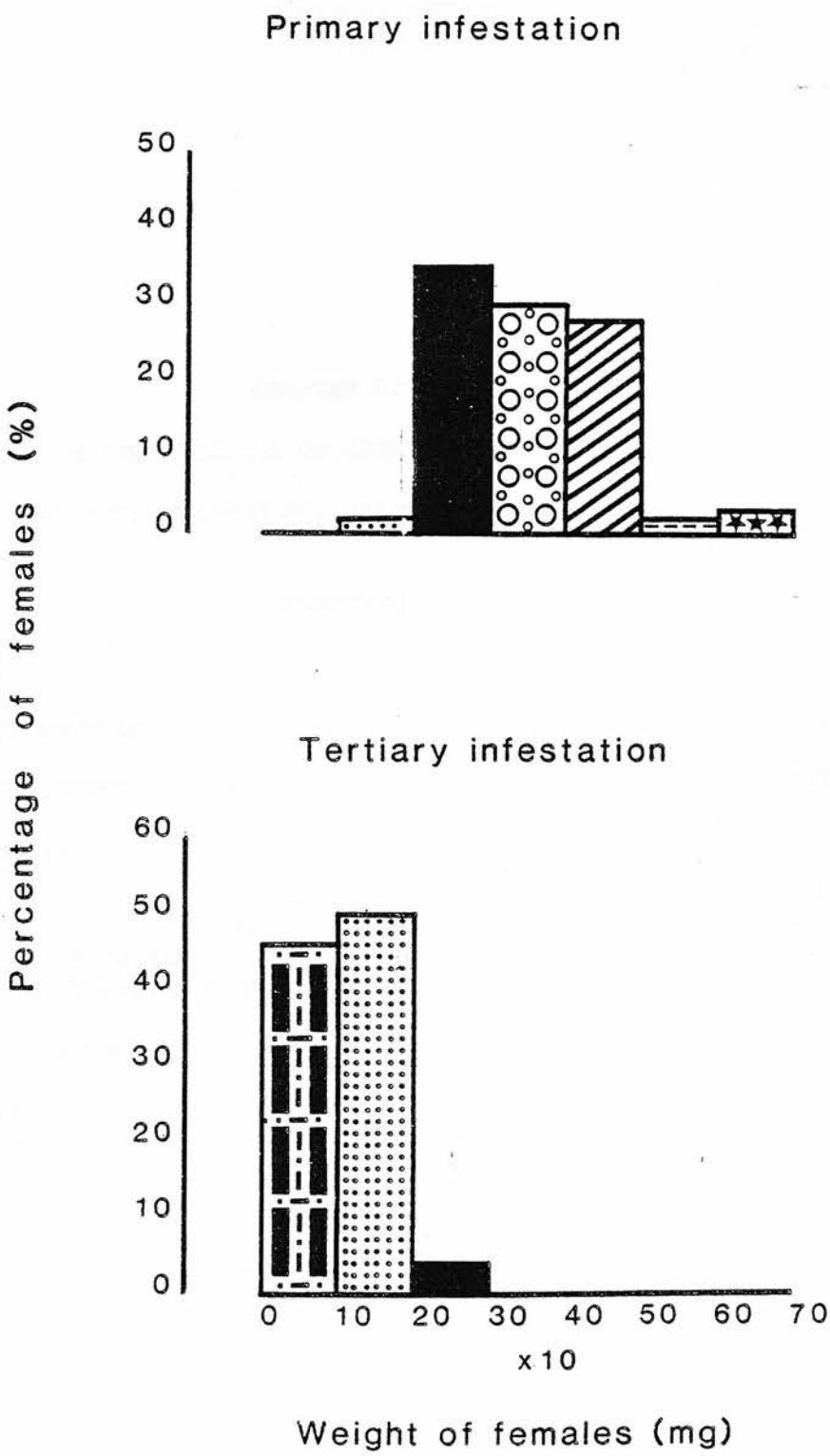

Mast-cells
Primary infestation
Tertiary infestation
20
15
10
5
0
Basophils
125
100
75
50
25
0
Eosinophils
40
32
24
16
8
0
x10
Neutrophils
100
80
60
40
20
0
x10
Mononuclear cells
50
40
30
20
10
0
x10
Number of cells/20 fields
0
24
48
72
96
120
144
Time after tick attachment (hours)

Figure 7.14 Frequency distribution of engorged weights of female H. a. anatolicum following primary and tertiary infestations on rabbits. (Class intervals are 100 mg).


Primary infestation
50
40
30
20
10
0
Tertiary infestation
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70
x10
Percentage of females (%)
Weight of females (mg)

CHAPTER EIGHT

HISTOPATHOLOGY OF TICK FEEDING SITES ON CATTLE DURING ACQUISITION OF RESISTANCE

CONTENTS

8.1 INTRODUCTION

The sequential histology of tick feeding sites has been the subject of active interest in recent years. All these studies except Tatchell and Moorhouse (1968) and Allen et al. (1977) have employed guinea-pigs and rabbits as hosts which are not the natural hosts of the tick in question. However, considerable useful information has accumulated from these studies. In guinea-pigs, basophils, and to a lesser extent, eosinophils were found to be the main immune effector cells of tick resistance (Brown et al., 1982a) and the level of tick rejection was related to the magnitude of tissue basophilia (Brown and Askenase, 1981). Basophils, were reported to be present in small numbers at tick feeding sites in rabbits (Brossard and Fivaz, 1982).

On the other hand basophils were rarely seen at B. microplus feeding sites on cattle (Schleger et al., 1976), a natural host-parasite situation. Whether or not this was due to lack of appropriate staining methods is not clear. In contrast basophils were present in significant numbers at I. holocyclus feeding sites in cattle (Allen et al., 1977). This presents a situation where it is difficult to make any generalizations.

In the light of the information available from laboratory animal-tick systems, it is very important to investigate more tick-cattle systems to evaluate these results and to see if these mechanisms are also active in natural host-parasite relationships.

Therefore, the present investigation was undertaken with the following objectives:

(i) To define and compare the sequence of histological changes at adult H. a. anatolicum tick feeding sites in naive and resistant bovine hosts.

(ii) To have an insight into immune cellular interactions detrimental to the tick.

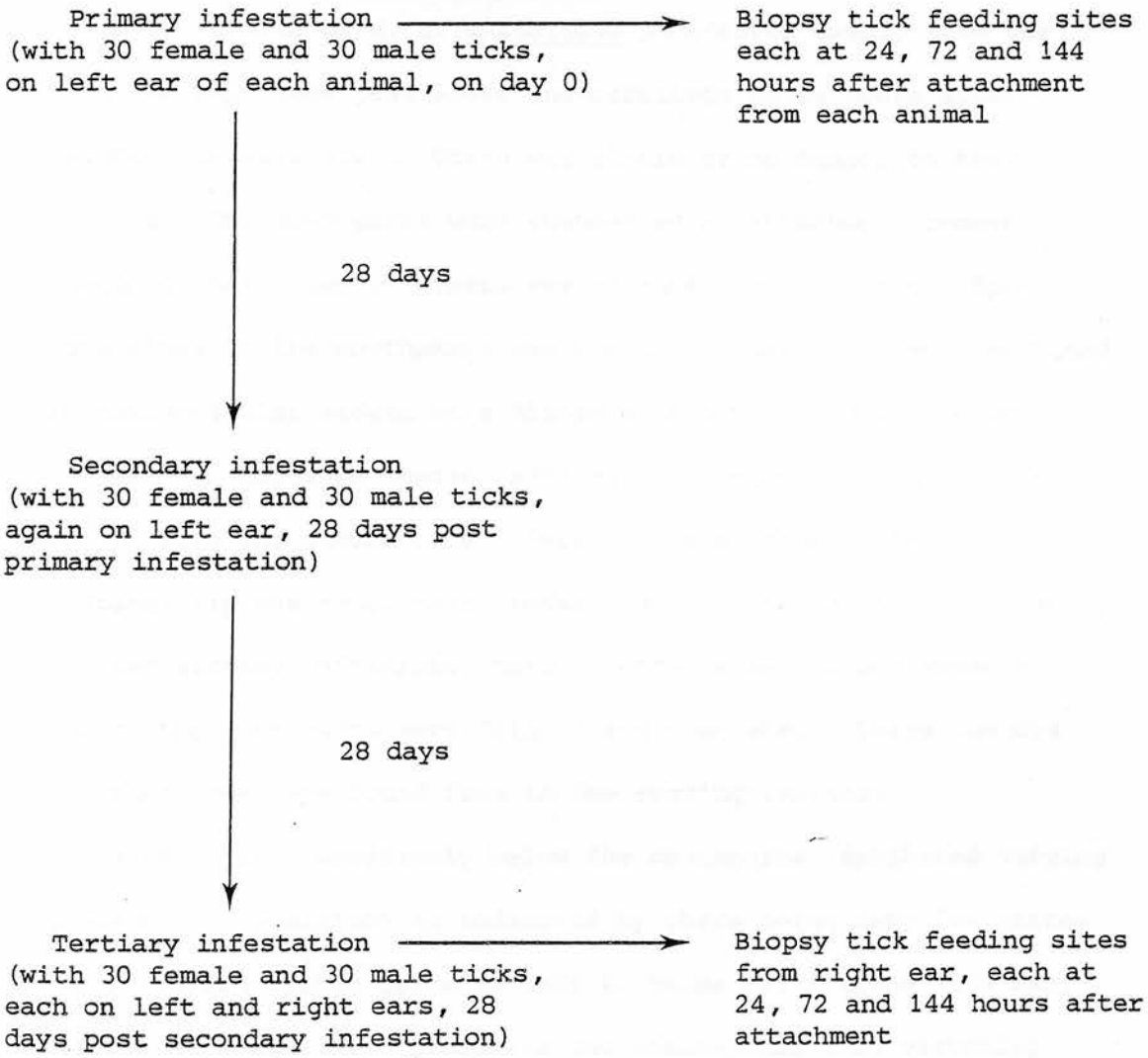
8.2 EXPERIMENTAL DESIGN

Four calves were subjected to tick infestation as scheduled in Figure 8.1. In each case excess ticks were removed after 24 hours to leave 17 females and 20 males in the primary, 10 females and 10 males in the secondary and 24 females and 35 males (17 females and 20 males on the right ear, and 7 females and 15 males on the left ear) in the tertiary infestation.

For histological analysis three female tick feeding sites with attached mouthparts were biopsied under local anaesthetic (2% Xylocaine) from each calf at 24, 72 and 144 hours after primary and tertiary attachments using a 3.5 mm trephine rotated rapidly with a hand-held electric motor. Two control biopsies were taken from each animal before primary and tertiary infestations i.e. on days 0 and 56. Biopsies were processed for quantification of histological responses as detailed in the general materials and methods.

The effect of resistance was monitored by recording and comparing the time to engorgement, numbers of ticks engorged, engorged weight, weight of egg mass and failure to lay eggs following primary and tertiary infestation.

Figure 8.1 Experimental design



8.3 RESULTS

8.3.1 Primary Infestation

Mouthparts of H. a. anatolicum penetrated deeply into the dermis and were seen just above the cartilage at 24 hours after attachment (Figure 8.2). There was little or no damage to the cartilage. The mouthparts were ensheathed by attachment cement throughout their length. There was no superficial cement. Epidermis close to the mouthparts was completely destroyed and exhibited mild intracellular oedema at a distance from the actual site of penetration. Collagen bundles adjacent to the mouthparts were seen embedded into the cement cone. Dermal tissue directly beneath the hypostomal tip was completely broken. A small number of inflammatory cells had already infiltrated this affected area. Blood vessels close to the mouthparts were dilated and congested. Large numbers of erythrocytes were found free in the feeding lesions.

Mast-cells, immediately below the mouthparts, exhibited varying degrees of degranulation as indicated by there being very few intracellular metachromatic granules left in these cells along with many extracellular granules. Basophils and eosinophils were virtually absent from all the feeding sites examined. Neutrophils (43%) dominated the inflammatory infiltrate followed by mononuclear cells.

At 72 hours after attachment the feeding lesion had almost doubled in size due to increased numbers and density of inflammatory cells. However, the histological picture had changed very little. Epidermis adjacent to the mouthparts displayed a moderate degree of oedema and hyperplasia. Some of the granulocytes below the mouthparts

and along the cement cone were undergoing necrosis. Increased capillary permeability had resulted in oedema and extravasation of a large number of erythrocytes.

The inflammatory infiltrate was more intense than that at 24 hours. Analysis of the cellular infiltrate revealed that neutrophils were the predominant leucocytes. Despite a marked increase in the absolute numbers of mononuclear cells, their proportion had fallen considerably due to an increased overall density of inflammatory cells (Tables 8.1 and 8.2).

Basophils appeared at some of the attachment sites but in fairly small numbers and formed 1% of the infiltrate (Table 8.2). Eosinophils were only seen at one out of 12 feeding sites examined (Appendix 3.2).

By 144 hours after attachment the inflammatory response had reached its maximum extent. The feeding lesion had further increased in size due to a substantial increase in infiltrating leucocytes. In a few biopsies the epidermis showed the formation of small epidermal vesicles close to the mouthparts. The epidermis showed oedema, hyperplasia and slight spongiosis in some biopsies. Accumulation and degeneration of neutrophils had resulted in collagen destruction at the tip of mouthparts and close to the attachment cement. Oedema and haemorrhages were of a greater degree and had resulted in an overall increased thickness of the dermis. Hair follicles and sebaceous glands were undamaged.

Basophil leucocytes were more common than at earlier stages. Eosinophil infiltration was also noticed in most of the biopsies. A few of these basophils and eosinophils immediately below the mouthparts exhibited a mild degree of degranulation.

Table 8.1 Cellular responses in the dermis of cattle at female H. a. anatolicum feeding sites

Infestation	Time after attachment (hours)	Numbers of cells/20 fields				
		Mast-cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
Primary (n = 12)	24	10+ (1-20)**	-	-	20 (4-372)	27 (8-125)
Tertiary (n = 12)	24	2.5 (0-11)	10.5* (1-333)	17.5* (1-94)	449* (236-1360)	143.5* (91-254)
Primary (n = 12)	72	6.5 (2-22)	0.5 (0-11)	- (0-2)	309.5 (59-640)	89.5 (47-211)
Tertiary (n = 12)	72	1.5* (0-19)	241* (31-686)	52.5* (14-146)	1253.5* (532-1510)	236.5* (109-442)
Primary (n = 12)	144	5 (1-12)	17.5 (0-44)	1 (0-6)	443 (257-1036)	185.5 (63-311)
Tertiary (n = 12)	144	1 (0-6)	542* (77-814)	72* (34-375)	730 (292-973)	356.5* (117-482)

+Median value; **Range of values; *Primary vs. Tertiary infestation ($P < 0.01$) Mann-Whitney's U test

Mononuclear cells included lymphocytes, monocytes and fibroblasts.

Addendum at end

Table 8.2 Mean proportions of mast cells, basophils, eosinophils, neutrophils and mononuclear cells in the dermis of cattle at female H. a. anatolicum feeding sites

Infestation	Time after attachment (hours)	Mast-cells %	Basophils %	Eosinophils %	Neutrophils %	Mononuclear cells %
Primary (n = 12)	24	22 (2-63)*	-	-	43 (12-90)	35 (7-57)
Tertiary (n = 12)	24	1 (0-3)	6 (0-36)	3 (0-36)	66 (42-83)	24 (12-46)
Primary (n = 12)	72	2 (1-7)	1 (0-4)	0 (0-1)	69 (47-81)	28 (15-35)
Tertiary (n = 12)	72	0 (0-1)	16 (2-45)	3 (1-6)	68 (37-87)	13 (6-22)
Primary (n = 12)	144	1 (0-1)	3 (1-5)	0 (0-1)	71 (57-81)	25 (19-41)
Tertiary (n = 12)	144	0 (0-<1)	23 (9-40)	9 (2-18)	48 (31-75)	20 (8-40)

n = number of observations
*Range of values
N.B. All the values are rounded up to the nearest whole number.

The dermal cellular infiltrate was composed predominantly of neutrophils, with large numbers of mononuclear cells and small numbers of basophils (Table 8.1). Mononuclear cells were more prominent at the periphery of the lesion.

8.3.2 Tertiary Infestation

At 24 hours after attachment epidermal vesicles had formed around all the attached ticks. These vesicles varied in diameter from 2-4 mm. Vesiculation was accompanied by an excessive serous exudation. There were many petechiae present suggesting attachment attempts by the ticks. A few vesicles were seen without ticks (Figure 8.3), probably in places where ticks had attached and were then forced to move to other feeding sites. Macroscopically the ears were swollen, hyperaemic and oedematous.

By 72 hours, most of the ticks were half drowned in the vesicles. However, the swelling and exudation of the ears was no longer present.

By 144 hours, all the ticks were entrapped in scabs formed from the vesicles and appeared to have ceased feeding. They were cream coloured and were between the first and second stages of feeding. The ticks which were fully drowned in the exudate were virtually dead by this time.

Histologically, 24 hours after tertiary attachment the feeding lesions were well developed and were comparable to 144 hour primary lesions. In general the inflammatory response was of a greater degree and all the histological changes observed during primary infestation were more intense.

The epidermis close to the mouthparts was completely replaced by the epidermal vesicles (Figures 8.4 and 8.5). The epidermis adjacent to these vesicles showed intercellular and intracellular oedema, hyperplasia and a moderate degree of vacuolization (Figure 8.6). The stratum germinativum was frequently disrupted by infiltrating neutrophils and basophils (Figure 8.7). Increased vascular permeability had resulted in dermal oedema. Haemorrhage was extensive (Figure 8.8).

Unlike the primary infestation the cellular infiltrate at this stage was characterized by the presence of large numbers of basophilic leucocytes along with small numbers of eosinophils. Basophils, along with tissue mast-cells, showed massive degranulation (Figure 8.9) especially beneath the mouthparts and along the attachment cement. Occasionally, eosinophils also showed a mild degree of degranulation. Neutrophils were the major component of the cellular infiltrate followed by mononuclear cells.

By 72 hours after attachment, the feeding lesion had further increased in size (Figure 8.10). The epidermal vesiculization was more pronounced. The epidermis showed marked oedema, hyperplasia and hyperkeratosis. The dermis infiltrate had doubled as compared to the 24 hour feeding lesions. Vascular damage was marked. Small areas of necrosis were seen along the side of the cement cone and at the tip of the mouthparts. Degranulation of mast-cells, basophils and eosinophils was of a much greater degree. Large numbers of metachromatic and eosinophilic granules were seen extracellularly. Mononuclear cells were more prominent towards the periphery of the lesion. Perivascular cuffing, mainly by eosinophils (Figure 8.11) with a few

mononuclear cells and occasionally a few basophils, was a characteristic feature of this feeding stage. Localized accumulations of eosinophils away from the mouthparts was also a striking feature. Intercellular oedema of the dermis was marked and had resulted in increased thickness of the dermis.

Analysis of the cellular infiltrate revealed that basophils had further increased in frequency and made up 16% of the total cells. The proportions of eosinophils and neutrophils were unchanged. Mononuclear cells showed a significant decrease and were only 13% of the infiltrate (Tables 8.1 and 8.2).

At 144 hours, epidermal vesiculation, oedema, hyperplasia, spongiosis and hyperkeratosis were all of a greater degree. Basophils and eosinophils exhibited massive degranulation. Necrosis of the dermis in the immediate vicinity of the mouthparts and along the cement cone was obvious and made it difficult to identify individual cells in this area. However, a large number of basophilic and eosinophilic granules were frequently seen.

Quantification of the cellular responses showed that the composition of the infiltrate had changed significantly by this time (Table 8.2). Basophils were the second most numerous cell type followed by mononuclear cells. Eosinophils had also greatly increased in number and formed 9% of the total (Tables 8.1 and 8.2).

Epidermal vesicles: Intraepidermal vesicles of varying size were noticed at all the tick attachment sites as early as 24 hours after tertiary infestation and at 144 hours following primary infestation in a few biopsies. In some biopsies the epidermis adjacent to

the mouthparts was completely replaced by these vesicles (Figure 8.4), whereas in others these were seen at a distance in between the stratum corneum and stratum granulosum (Figure 8.7). These vesicles were primarily composed of neutrophils with a moderate number of basophils and debris of epidermal cells (Figure 8.5). Eosinophils and mononuclear cells were rare. In some sections very small vesicles were observed in addition to the big vesicles further away from the mouthparts. Epidermal cells in different stages of degeneration and inflammatory cells breaking through the dermo-epidermal junction were clearly seen in these vesicles.

By 72 to 144 hours, most of the inflammatory cells in these vesicles had degenerated and all the vesicles appeared like small abscesses.

8.3.3 Kinetics of Individual Cell Types

Mast-cells: The mean numbers of mast-cells observed in the control biopsies ranged from 6-46 before primary, and 26-63 before tertiary infestation. Five of the eight biopsies taken before tertiary infestation showed a large increase in the numbers of mast-cells (Appendix 3.1).

Following primary infestation their numbers decreased progressively as the feeding advanced. At 144 hours the mast-cells present at the dermo-epidermal junction, close to the attachment site and directly below the mouthparts contained very few metachromatic granules.

There was a rapid decrease in the numbers of mast-cells following tertiary infestation (Figure 8.12) and they were significantly

lower at 72 and 144 hours compared to the primary infestation (Table 8.1). At 144 hours, they were completely absent from 50% of the biopsies examined (Appendix 3.3).

Basophils: The number of basophils observed during the primary and tertiary infestations and their trend during feeding have been presented in Tables 8.1 and 8.2 and Figure 8.12. No basophils were observed in control biopsies and the feeding sites biopsied at 24 hours post primary attachment. They were first observed at 72 hours at which time 41% of the biopsies had small numbers of basophils. Thereafter, they showed progressive increase in numbers and made up to 3% of the cellular infiltrate at 144 hours (Tables 8.1 and 8.2).

In contrast to primary infestation basophils were observed in significantly greater numbers as early as 24 hours after tertiary attachment. They further increased in absolute numbers and proportions as the feeding advanced. Peak tissue basophilia was observed at 144 hours (Figure 8.12) at which time they formed 23% of the cellular infiltrate. They were the second most abundant cell types after neutrophils at 72 hours and 144 hours (Table 8.2). The actual numbers of basophils infiltrating tertiary feeding sites might be much higher than recorded as many of these degranulate as soon as they enter the feeding site, thus making it difficult to accurately monitor the magnitude of the basophil response.

Eosinophils: These were virtually absent from the control biopsies. Two of the eight biopsies taken before tertiary infestation had varying numbers of eosinophils (Appendix 3.1). Following primary

infestation eosinophils were never present in the cellular infiltrate at the earlier observation times. They were completely absent from all the biopsies at 24 and 72 hours, except in one biopsy which had a few eosinophils. They were more commonly seen at 144 hours after attachment (Appendix 3.3).

Eosinophils were much more abundant following tertiary infestation. They formed 3% of the cellular infiltrate at 24 hours (Table 8.2). As the feeding advanced they increased in their frequency reaching a maximum at 144 hours (Figure 8.12), at which time they constituted 9% of the cellular infiltrate.

Neutrophils: Neutrophils were the most numerous cell types observed throughout primary and tertiary feedings (Table 8.2). On primary feedings they were present in small to moderate numbers up to 72 hours and showed a dramatic increase in absolute numbers thereafter, reaching peak levels (71%) at 144 hours.

Following tertiary attachment they were present in significantly greater numbers (Table 8.1) exhibiting peak levels at 72 hours (Figure 8.12) and then showing a gradual decrease in numbers as well as proportions. At 144 hours, they were 48% of the cellular infiltrate.

Mononuclear cells: Mononuclear cells were the second most common cell type throughout primary infestation and formed 25% to 35% of the infiltrate (Table 8.2).

On tertiary infestation they were the second most predominant cells up to 24 hours, when they formed 24% of the total cells. Despite increased numbers their proportion fell significantly and they were 13% of the cellular infiltrate at 72 hours.

8.3.4 Expression of Resistance

The effect of resistance on feeding, egg production and egg hatching has been summarised in Table 8.3. The distribution pattern of fed weights following primary and tertiary infestation is presented in Figure 8.13. The acquisition of resistance was expressed by length and time to engorgement, reduced engorgement weight, decreased egg production, failure to lay eggs and in many instances death *in situ*.

During primary infestation ticks took an average of 6.5 days to complete engorgement and this period was increased to 7.6 days for those ticks that succeeded on tertiary infestation. Fifty-seven percent of the females failed to engorge and died on the host during the tertiary infestation.

Female ticks fed on naive hosts weighed 457.7 mg on average. On tertiary infestation the average fed weight fell dramatically to 89.6 mg which was only 20% of the primary fed weights (Table 8.3).

The mean weight of egg masses following primary infestation was 268 mg and was 59% of the fed weight. The mean weight of the egg mass dropped significantly following tertiary infestation and was only 31% of the fed weight. Six percent of the females on primary and 54% on tertiary infestation failed to lay eggs. However, all the eggs laid following primary infestation hatched normally whereas eggs laid by 40% of females on tertiary infestation failed to hatch.

Table 8.3 Effect of resistance on feeding and egg laying in female H. a. anatolicum

	Primary infestation	Tertiary infestation
Mean time to engorgement (days)	6.5 ± 0.75* (n = 34)	7.6 ± 0.9 (n = 13)
Mean weight of engorged females (mg)	457.7 ± 81.6 (n = 34)	89.6 ± 65.9**(n = 13)
Mean weight of egg mass (mg)	267.97 ± 58.6 (n = 32)	54.6 ± 52.0**(n = 5)
Percent eggs laid of engorged weight	58.8	31.0
Failure to lay eggs (%)	5.9	61.5
Failure of egg hatching (%)	-	40.0
Death in situ (%)	-	56.7

*Mean ± standard error; n = number of observations

**Primary vs. Tertiary ($P < 0.01$)

8.4 DISCUSSION

After tertiary infestation the formation of epidermal vesicles was observed as early as 24 hours. This was accompanied by excessive serous exudation, hyperaemia and oedema of the ears. Similar types of epidermal lesions have been reported in cattle resistant to B. microplus (Tatchell and Moorhouse, 1968) and I. holocyclus (Allen et al., 1977) and in guinea-pigs resistant to D. andersoni (Trager, 1939a; Allen, 1973), A. americanum (Brown and Knap, 1981) and R. appendiculatus (Brown et al., 1983). On the contrary, epidermal lesions were completely absent in dogs repeatedly infested with R. sanguineus (Theis and Budwiser, 1974). This is interesting in the context that dogs never develop resistance to R. sanguineus. The ability to produce epidermal vesicles reflected the resistance status of the host (Trager, 1939a; Tatchell and Moorhouse, 1968). Vesiculation, followed by exudation and later encrustation resulted in poor engorgement and sometimes death of the ticks in situ.

Microscopically, the epidermal vesicles were primarily composed of neutrophils but also had small numbers of basophils, which is in contrast to the basophil-rich epidermal vesicles found at tick feeding sites in resistant guinea-pigs (Allen, 1973; Bagnall, 1978; Wikel and Allen, 1977; Brown and Askenase, 1981; Brown et al., 1983; Brown et al., 1984).

The dermal cellular infiltrate was dominated by neutrophils throughout primary and tertiary infestations (Table 8.2). This could be purely an inflammatory response of the host to tissue insult. In addition, the salivary secretions might have contributed by generating chemotactic factors for neutrophils (Berenberg et al., 1972). However,

the greater magnitude of the neutrophil infiltrate following tertiary infestation could be due to the formation of immune complexes (Cochrane, 1971) following interaction between host immunoglobulins and tick salivary antigens. Degeneration of neutrophils corresponded with the degree of tissue destruction at the tick feeding sites and was in agreement with the observations of Tatchell and Moorhouse (1970), and Theis and Budwiser (1974). However, the cytolytic effect of the salivary secretions in causing tissue destruction (Kemp et al., 1982) cannot be discounted.

The observed increase in the mast-cell numbers in the control biopsies before tertiary infestation, not confined only to the ear subjected to tick infestation but also on the opposite ear, was intriguing. The small sample size and the differences between individual animals makes it difficult to come to a definite conclusion. Further observations are therefore required on the mast-cell response before and after tick infestations.

Mast-cell degranulation following primary infestation could be due to mechanical disruption of cells, and salivary secretions (Geczy et al., 1971). However, the mast-cell degranulation could also be due to activation of complement during acute inflammation (Movat, 1971). Basophil infiltration at 72 hours after primary infestation could be mediated by sensitized T cells (McLaren et al., 1983).

Following tertiary infestation with H. a. anatolicum basophils infiltrated tick feeding sites in significantly greater numbers and made up 23% of the cellular infiltrate at 144 hours. In contrast

basophils were rarely seen at B. microplus feeding sites in cattle and were present in small proportions at tick feeding sites in resistant rabbits (Brossard and Fivaz, 1982; 7.3.1.2). On the other hand basophils were the predominant cells at I. holocyclus feeding sites in cattle one hour after tertiary attachment, but showed a dramatic decrease thereafter, and were only 12% of the cellular infiltrate at 12 hours (Allen et al., 1977). Unlike the I. holocyclus-cattle system, basophils in this study showed a progressive increase in absolute numbers as well as proportions and were the second most abundant cell types at 72 and 144 hours following tertiary infestation. A similar trend, although greater in magnitude, has been reported in the guinea-pig-tick host system (Allen, 1973; Bagnall, 1978; Brown and Knap, 1981; Askenase et al., 1982; Brown et al., 1984). The recruitment of basophils to tick feeding sites is a T cell (Wikel, 1976) antibody (Brown and Askenase, 1981; Askenase et al., 1982 Brown et al., 1984) and complement (Wikel and Allen, 1977) -dependent immune mechanism.

The differences observed in the magnitude of the basophil response in individual animals (Appendix 8.3) could be due to mast-cell density in the skin of the host (Schleger et al., 1976), differences in serum IgE levels and the ability of mast-cells to bind IgE (Willadsen et al., 1979). The greater degree of mast-cell and basophil degranulation on tertiary infestation could be the result of interaction between salivary antigens of the tick and specific homocytotropic antibodies bound to the surface of mast-cells and basophils. McLaren et al. (1983) reported that this interaction triggered a series of intracellular events which ultimately resulted

in anaphylactic degranulation. The degranulation close to the attachment cement could be due to antigenic aminopeptidase present in the cement cone of H. a. anatolicum (5.3.2.3 and 6.3).

Eosinophils closely followed basophils and were first noticed at tick feeding sites, 144 hours after primary attachment. This could be due to the chemoattractants released by degranulating mast-cells. On tertiary infestation eosinophils infiltrated tick attachment sites in small numbers by 24 hours and exhibited mild degranulation. The accumulation of eosinophils could be due to chemoattractants in saliva (Berenberg et al., 1972), factors released by degranulated mast-cells and basophils, sensitized T cells (Wikel, 1976) and IgG antibodies (Brown et al., 1982b). The kinetics of eosinophils differed significantly from those at B. microplus (Schleger et al., 1976; Schleger et al., 1981) and I. holocyclus feeding sites on cattle. Eosinophils were the predominant cells at I. holocyclus feeding sites in cattle at four hours after tertiary infestation and formed more than 29% of the infiltrate at 12 hours, and were present in significant numbers at B. microplus feeding sites. The degree of resistance to B. microplus was proportional to mast-cell density, infiltration and degranulation of eosinophils (Schleger et al., 1976).

Basophil and mast-cell granules are a rich source of histamine and 5-hydroxytryptamine (Askenase, 1977). Eosinophils modulate the effect of histamine (Weller and Goetzl, 1979) and have even been shown to translocate histamine to the tick attachment sites (Schleger et al., 1981). Thus, the degranulation of basophils and mast-cells results in increased levels of histamine and 5-hydroxytryptamine believed to be necessary for mediating tick resistance and rejection.

There is considerable evidence to support this view:

(i) Treatment of resistant hosts with antihistamines greatly inhibited the expression of resistance (Tatchell and Bennett, 1969; Brossard, 1982; Wikel, 1982).

(ii) Willadsen *et al.* (1979) and Wikel (1982) found increased histamine levels in the skin of resistant hosts and Riek (1962) reported an increased level of blood histamine in cattle resistant to *B. microplus*.

(iii) Addition of histamine and 5-hydroxytryptamine into the feeding medium of *D. andersoni* ticks in an *in vitro* system significantly reduced the recordings associated with feeding (Paine *et al.*, 1983).

(iv) Presence of antihistamines in tick salivary glands (Chinery and Ayitey-Smith, 1977).

However, the exact mechanism by which these factors, released during immune interactions, mediate resistance is not yet known.

One of the possible mechanisms is that the histamine released from degranulated mast-cells and basophils at tick feeding sites causes pruritis and stimulates a reflex grooming response by the host (Schleger *et al.*, 1976), which has been found to be the major cause of tick losses (Koudstaal *et al.*, 1978). However, host grooming does not appear to be the only effector mechanism. In the restrained grooming experiments of Bennett (1969) only 9.2% of the ticks engorged, indicating that there are other mechanisms involved in the expression of resistance.

Secondly, from the *in vitro* studies of Paine *et al.* (1983) and the authors own observations on the salivary glands of ticks fed on resistant hosts, one of the possible effector mechanisms involved in the expression of resistance appears to be the direct effect of histamine and 5-hydroxytryptamine on the mechanisms controlling salivary secretions. How this effect is achieved is open to speculation. It is possible that these amines compete (antagonists or very weak agonists) with catecholamines at neuro-secretory junctions or may act on chemoreceptors at the cheliceral digits (Waladde and Rice, 1978) and deprive the tick of salivary enzymes vital for feeding. In addition, these amines to some extent inhibit digestion by altering the structure or activity of some of these salivary enzymes and may also cause the death of the parasite *in situ* by damaging some vital physiological centres.

Thirdly, the specific antibodies demonstrated at tick feeding sites (Allen *et al.*, 1979) might inactivate the bulk of the salivary enzymes essential for successful feeding.

The acquisition of resistance by cattle resulted in prolonged times to engorgement and poor nutrition. The fed weights of the ticks on tertiary infestation were significantly lower than those of ticks fed in the primary infestation (Table 8.3). A significant proportion (40%) of the females failed to complete engorgement and died *in situ*. This could be due mainly to their being entrapped in the exudate and might also be due to the toxic effect of pharmacological mediators ingested during feeding. Failure of a significant proportion of female ticks to lay eggs could be entirely due to poor nutrition or lack of fertilization as the vesicles around the mouthparts

of females had made it difficult for males to attach close to them. However, a marked decrease in eggs laid as a percentage of fed weight on tertiary infestation is strongly suggestive of a direct effect of pharmacological mediators on oviposition.

8.5 SUMMARY

A valuable insight into the nature of cellular interactions involved in the expression of resistance has been gained by comparing the tissue changes and cellular responses following primary and tertiary infestation by female H. a. anatolicum ticks on cattle. On primary infestation the cellular infiltrate was dominated by neutrophils (43-71%) followed by mononuclear cells (25-35%). A small number of basophils and eosinophils infiltrated tick feeding sites by 144 hours after attachment.

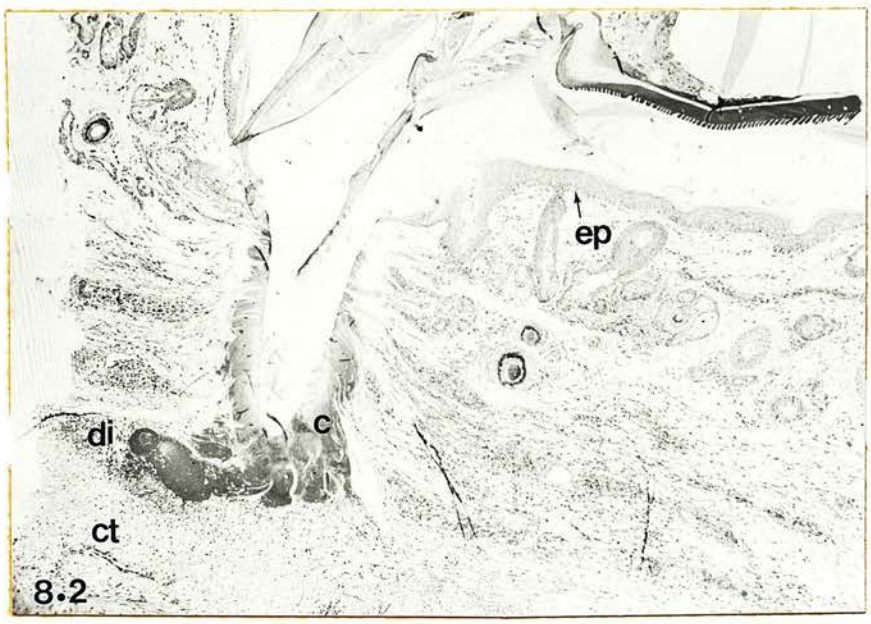
Following tertiary infestation, there was pronounced epidermal vesiculation accompanied by serous exudation, leading to entrapment of ticks which eventually resulted in poor feeding or death in situ. Histologically, the cellular infiltrate was typical of cutaneous basophil hypersensitivity reactions. Basophils formed 23% of the cellular infiltrate at 144 hours after attachment and were the second most abundant cell type after neutrophils (48%). Eosinophils and mononuclear cells also showed a significant increase in mean numbers as compared to primary infestation. The mast-cell disruption and infiltration, and degranulation of basophils were of a greater degree than observed during primary infestation.

The pharmacological mediators released by degranulating mast-cells and basophils appeared to be the major effectors of resistance in cattle to H. a. anatolicum ticks.

Figure 8.2 Primary infestation, 24 hours after attachment of female H. a. anatolicum to the ear of a cow. Methacrylate section (Giemsa, x 44).

Figure 8.3 Tertiary infestation, 24 hours after attachment of H. a. anatolicum to the ear of a cow. Note epidermal vesicles of varying sizes, with and without (arrows) ticks.

c = cement cone; ct = cartilage; di = dermal infiltration; ep = epidermis; ep.v = epidermal vesicle.



8.2

8.3

Figure 8.4 Tertiary infestation, 72 hours after attachment of female H. a. anatolicum to the ear of a cow. An epidermal vesicle adjacent to the mouthparts of the tick. Methacrylate section (Giemsa, x 109).

Figure 8.5 An epidermal vesicle (Figure 8.4) at a higher magnification. Note the composition of the vesicular infiltrate. Methacrylate section (Giemsa, x 1,088).

b = basophils; ep = epidermis; ep.v = epidermal vesicle; h = hypostome; m = mononuclear cells; n = neutrophils; st.c = stratum corneum.


h
ep.v
8.4
ep

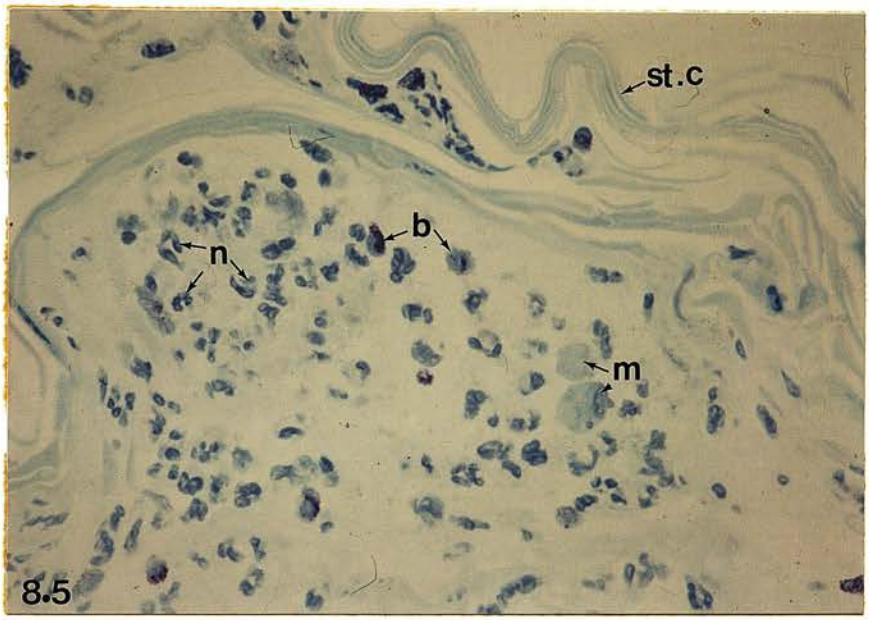

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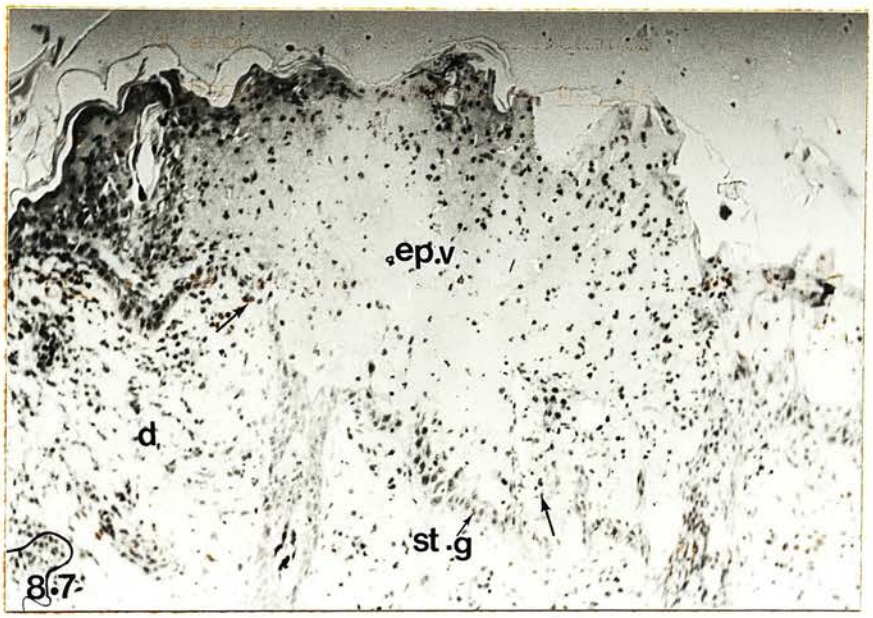
Figure 8.6 Tertiary infestation, 72 hours after attachment of female H. a. anatolicum to the ear of a cow. The epidermis at a distance from the mouthparts of the tick. Methacrylate section (Giemsa, x 544).

Figure 8.7 Tertiary infestation, 72 hours after attachment of female H. a. anatolicum to the ear of a cow. An epidermal vesicle at a distance from the mouthparts of the tick. Note the stratum germinativum is frequently disrupted by infiltrating leucocytes (arrows). Methacrylate section (Giemsa, x 272).

d = dermis; ep.v = epidermal vesicle; st.g = stratum germinativum.


ep.v
8.6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d
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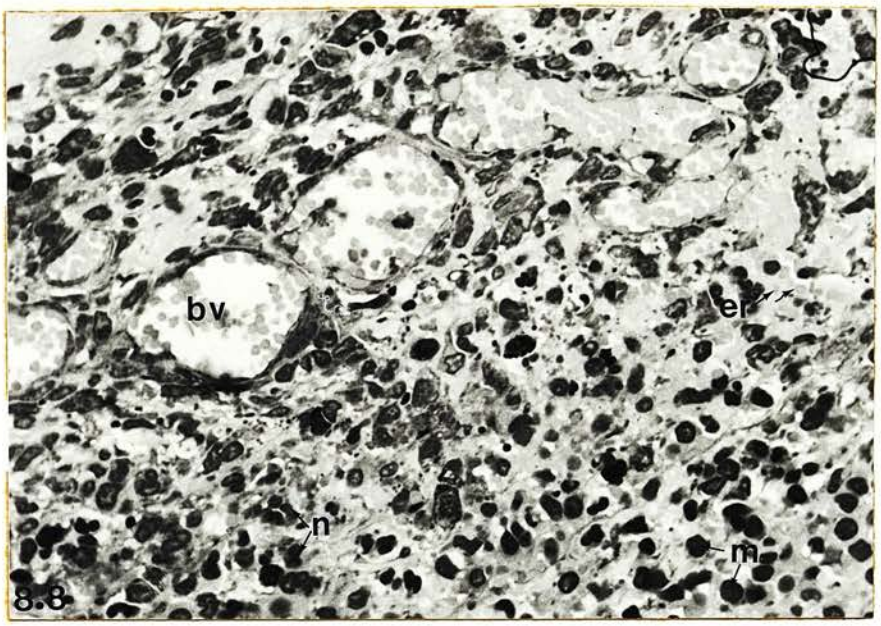
8.7

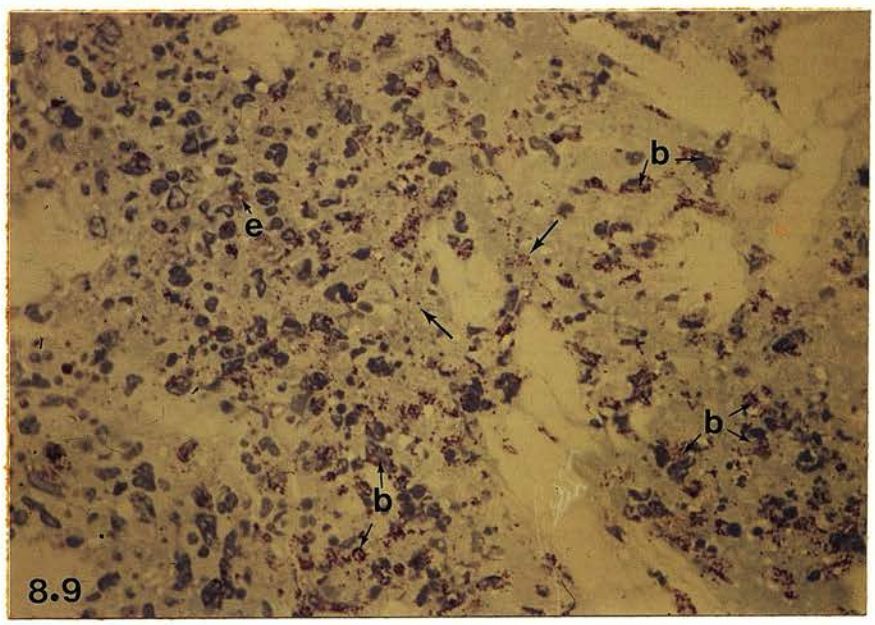
Figure 8.8 Tertiary infestation, 24 hours after attachment of female H. a. anatolicum to the ear of a cow. The dermis at a distance from the mouthparts of the tick. Methacrylate section (Giemsa, x 544).

Figure 8.9 Tertiary infestation, 24 hours after attachment of female H. a. anatolicum to the ear of a cow. The dermis immediately below the mouthparts of the tick. Note a large number of free metachromatic granules (arrows). Methacrylate section (Giemsa, x 544).

b = basophils; bv = blood vessel; e = eosinophils; er = erythrocytes; m = mononuclear cells; n = neutrophils.



8.8

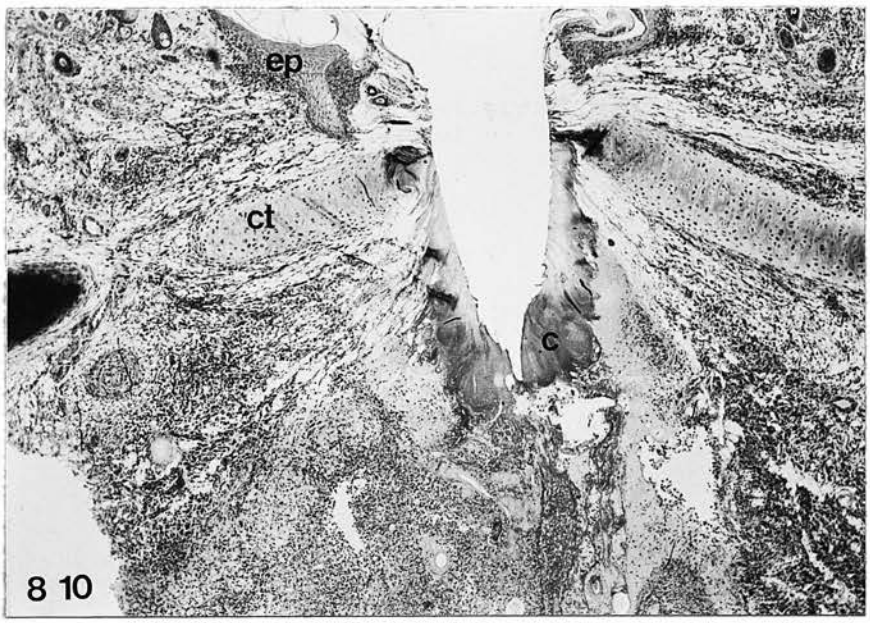


8.9

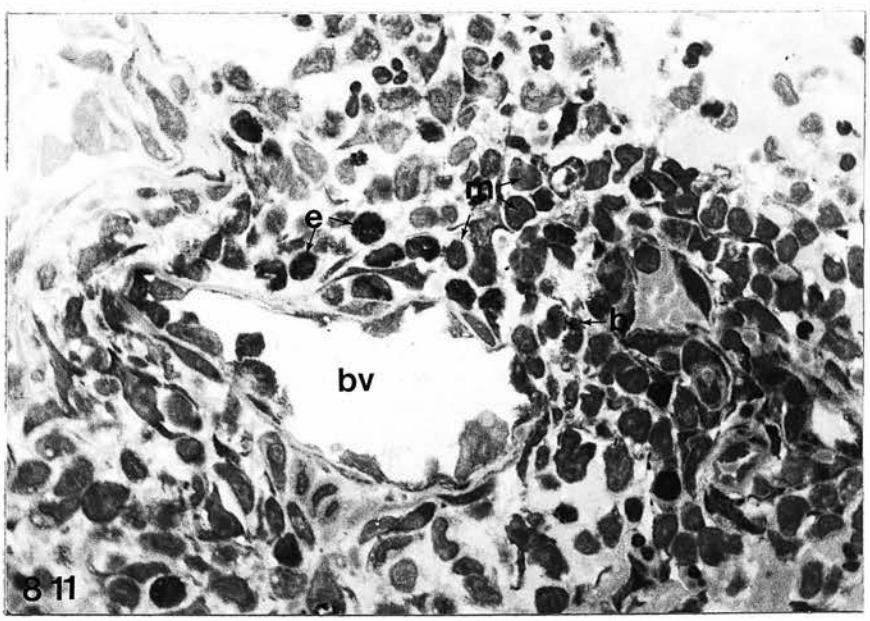
Figure 8.10 Tertiary infestation, 72 hours after attachment of female H. a. anatolicum to the ear of a cow. Methacrylate section (Giemsa, x 44).

Figure 8.11 Tertiary infestation, 72 hours after attachment of female H. a. anatolicum to the ear of a cow. The dermis at a distance from the mouthparts of the tick. Methacrylate section (Giemsa, x 1088).

b = basophil; bv = blood vessel; c = cement cone;
ct = cartilage; di = dermal infiltrate; e = eosinophils;
ep = epidermis; m = mononuclear cells.



8 10



8 11

Figure 8.12 Kinetics of cellular responses in the dermis of cattle at female H. a. anatolicum feeding sites. Each point represents the median of 12 observations.

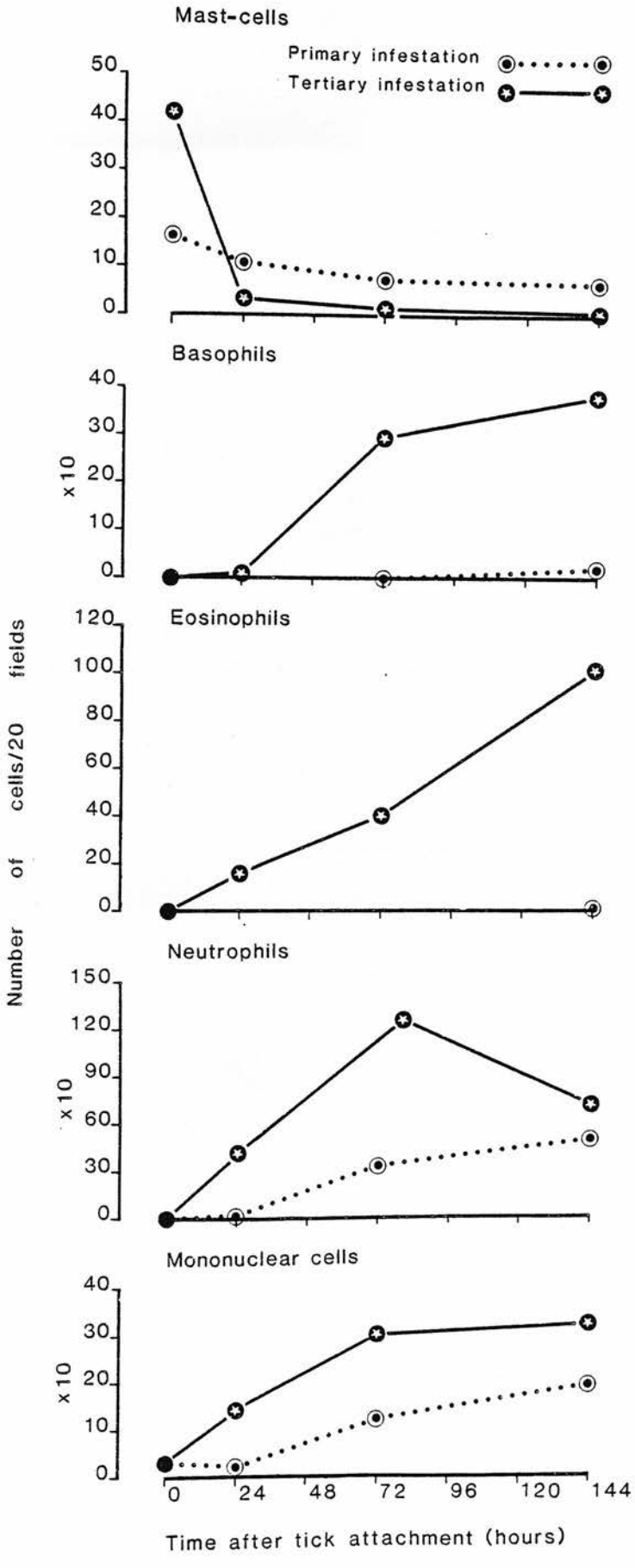
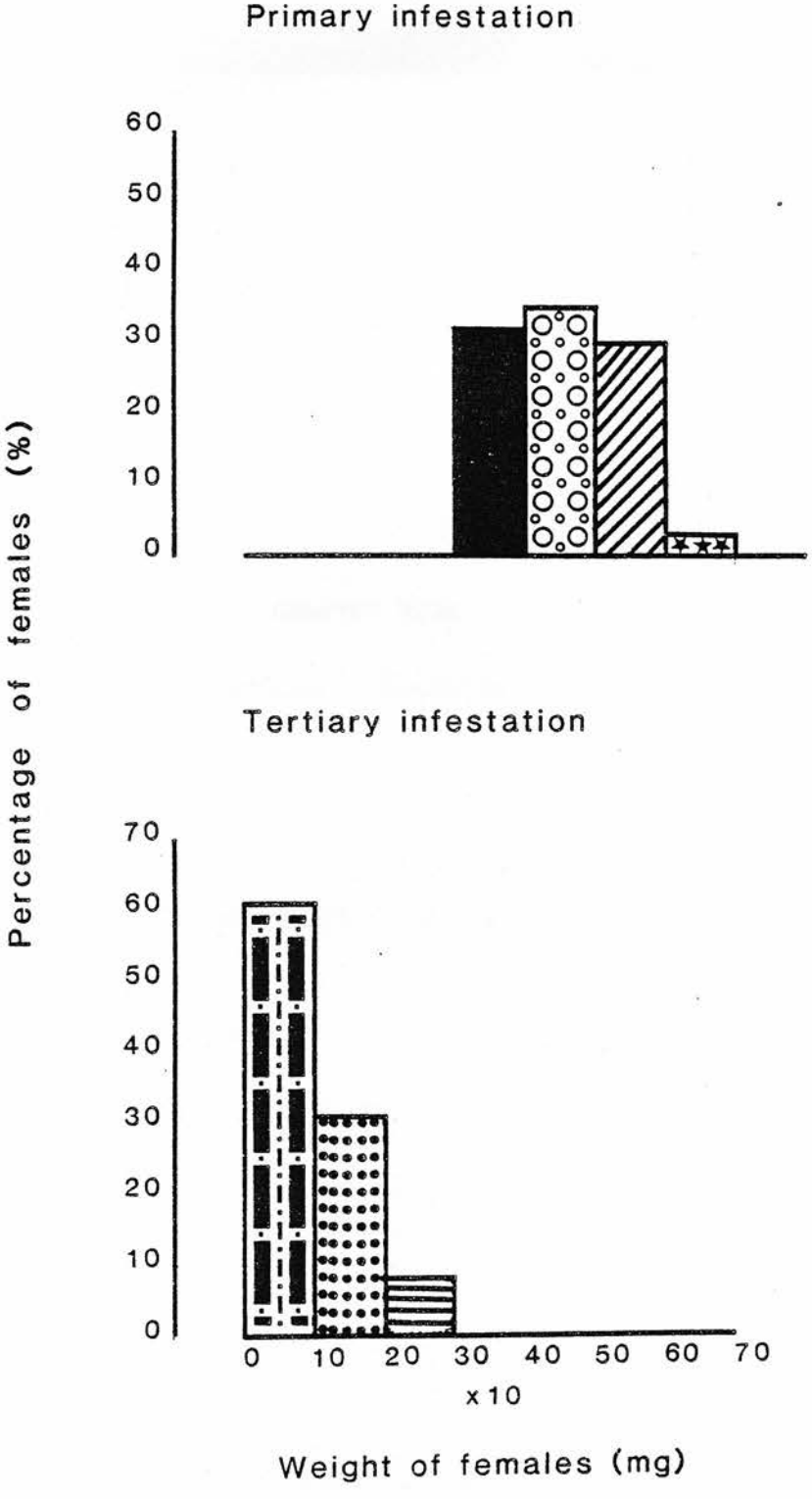

Mast-cells
Primary infestation
Tertiary infestation
50
40
30
20
10
0
Basophils
40
30
x10
20
10
0
Eosinophils
120
100
80
60
40
20
0
Number of cells/20 fields
Neutrophils
150
120
90
x10
60
30
0
Mononuclear cells
40
30
x10
20
10
0
0
24
48
72
96
120
144
Time after tick attachment (hours)

Figure 8.13 Frequency distribution of engorged weights of female H. a. anatolicum following primary and tertiary infestation on cattle (Class intervals are 100 mg).


Primary infestation
60
50
40
30
20
10
0
Tertiary infestation
70
60
50
40
30
20
10
0
0 10 20 30 40 50 60 70
x 10
Percentage of females (%)
Weight of females (mg)

CHAPTER NINE

GENERAL DISCUSSION

The range of interactions between ticks and their hosts are varied. Ixodid ticks feed on the host for several days to obtain a blood meal. In the process of feeding they secrete a variety of enzymes, anticoagulants, complement activators and inhibitors and pharmacological substances and their inhibitors. It is therefore reasonable to assume that many of these will be important antigens. This is apparent from the fact that laboratory animals and cattle develop resistance to tick feeding after a single infestation. The resistance is manifested as protracted feeding, reduced reproduction or death of the parasite on the host without engorgement. In natural situations ticks feed on hosts that have already been exposed to ticks and face a wide range of host responses (Willadsen, 1980). So while considering the function and importance of salivary secretions in feeding one must also take into account the type of host response the tick encounters while feeding.

The life cycle of ixodid ticks consists of short parasitic and extended non-parasitic phases without any access to food. During non-parasitic phases when the atmospheric humidity is low ticks are faced with the problem of maintaining their water balance to avoid dehydration. To cope with this inimical situation ticks have the remarkable ability to absorb water vapours from unsaturated air.

The type I acini in the salivary glands of H. a. anatolicum have shown ultrastructural features characteristic of fluid transporting epithelia (Pease, 1956). The well developed basal labyrinth, richly supplied with mitochondria and a strong ATP-ase activity in this region, supported the hypothesis of McMullen et al. (1976) that the

type I acini secrete concentrated salts during questing stages to absorb water from subsaturated environments.

At the onset of feeding the mouthparts of H. a. anatolicum penetrated deeply into the host skin and secured its attachment by the secretion of cement like other ixodid ticks, except some Prostriata (Kemp et al., 1982). The cement of H. a. anatolicum was lipoprotein in nature and appeared to have been derived from a cells of type II acini and d and e cells of type III acini, the secretory granules of which showed similar histochemical properties. However, in contrast to B. microplus (Moorhouse and Tatchell, 1966), and H. spinigera (Chinery, 1973) the cement cone of H. a. anatolicum did not reveal distinct cortex and internum layers. Unlike B. microplus, the cement was negative for polysaccharides.

The presence of aminopeptidase and acid-phosphatase in the cement cone, and the antigenic nature of these enzymes (6.3.3) suggested that they were secreted into the host as active constituents of salivary secretions and might have some important physiological significance. Aminopeptidase might be responsible for collagen homogenisation observed all along the cement cone, in order to prevent a foreign body type reaction immediately next to the cone. The acid phosphatase on the other hand might have some glueing function in the cone as suggested by Walker et al. (1984). In addition, the aminopeptidases have been considered among digestive enzymes in several haematophagous arthropods (Gooding, 1975). It was difficult to pinpoint the origin of aminopeptidase in the salivary glands as all the acinus types showed moderate to strong reactions for this enzyme.

The bulk of acid phosphatase could be from a cells of type II and d cells of type III acini, the secretory granules of which showed strong reactions for acid phosphatase.

The main function of the cement is to ensure firm attachment and prevent damage to adjacent dermal tissues (Moorhouse, 1969). However, it is interesting in the context that there are some species of Ixodes genus (with long mouthparts) which feed successfully without secretion of cement. It is possible that the deep insertion of mouthparts, supported by attachment cement allows H. a. anatolicum to inflict greater host tissue destruction by salivary enzymes as compared to Ixodes.

After having established secure attachment, ticks secrete a wide range of salivary agents into the host to ensure a constant supply of blood to complete engorgement. The localization of secretion of glycoproteins at tick feeding sites and the glycoprotein nature of all the antigens identified from saliva of H. a. anatolicum (6.3.3) suggested their physiological significance during feeding. It seemed likely that these secretions had originated from b and c cells of type II acini, the secretory granules of which showed strong glycoprotein activity. In addition, the b and c cells appeared to synthesize and secrete their products throughout feeding and appeared to be suitable candidates for various substances that have been associated with tick saliva in manipulating host responses during feeding. These include anticoagulants (Ross, 1926; Balashov, 1972), inflammatory agents (Dickinson et al., 1976; Shemesh et al., 1979), enzyme inhibitors (Willadsen and Riding, 1980), antihistamines (Chinery and

Ayitey-Smith, 1977), chemoattractants for neutrophils (Berenberg et al., 1972) and immunosuppressants (Wikel, 1981).

The bulk of esterase material identified at H. a. anatolicum feeding sites might have originated from b, c_1 and c_3 cells of type II acini, the granules of which showed moderate to strong reactions for esterases. The finding that one of the antigens (antigen III, molecular weight 130,000 daltons) identified from the saliva had an esterase activity confirmed that esterases were secreted into the host. The possibility that esterases might increase vascular permeability by their direct action on mast-cells (Geczy et al., 1971) or by inducing the formation of plasma kinins (Movat, 1971) was substantiated by the fact that antigen III (having an esterase activity) elicited an immediate oedematous reaction on intradermal inoculation into a rabbit with no previous exposure to ticks. This also explains the mast-cell degranulation observed at H. a. anatolicum feeding sites in cattle and rabbits on primary infestation. The rapid removal of esterases from tick feeding sites in hosts which rejected ticks (Tracey-Patte, 1979) suggested that they have a positive role for successful feeding. Of the three allergens isolated and purified from larval B. microplus ticks, two are known to have important physiological functions (Willadsen and Williams, 1976; Willadsen et al., 1978; Willadsen and Riding, 1979). One of these allergens was an esterase, whilst the other was a proteolytic enzyme inhibitor which also blocked the action of complement (Willadsen, 1980). All these lines of evidence suggest that these enzymes have important roles in feeding.

When ticks are feeding they ingest large quantities of blood, tissue fluids and ions with the blood meal. In order to concentrate nutrients and maintain ionic regulation they have to excrete excess fluids and ions. The ixodid salivary glands play an important role in excreting excess water during feeding. At the onset of feeding the inconspicuous interstitial cells become prominent to form a part of the water excretory unit. In H. a. anatolicum the interstitial cells were present in two tiers: a single adlumenal interstitial cell which extended all along the lumen alternating with granular cells, and several ablumenal interstitial cells which extended between the interstices of granular cells having no direct contact with the lumen.

During feeding the ablumenal interstitial cells of type III acini in females enlarged enormously to form a labyrinthine system of extraordinary complexity by interdigitating with the basolateral membranes of transformed f cells. This resulted in the formation of an extensive network of extracellular channels facing the haemolymph to excrete excess fluids during feeding as suggested by Meredith and Kaufman (1973) and Megaw and Beadle (1979). The occurrence of a strong ATP-ase activity, highly localized along the lumenal brush border and the interstitial cells, further confirmed their active involvement in osmoregulation. The interstitial cells in other acinus types might excrete small quantities of fluids to wash the secretions from secretory cells down to the main duct.

Following initial penetration a small feeding lesion was established immediately below the mouthparts caused by mechanical disruption of dermal collagen. The lesion extended rapidly due to

infiltration by inflammatory cells as a result of vascular damage inflicted by salivary secretions. The vascular and tissue damage noticed 24 hours after attachment might have been in part due to the secretion of weakly hydrolytic enzymes like aminopeptidase and non-specific esterase into the tissues of the host. However, the extensive tissue destruction observed during later stages of feeding could be attributed to the host response to salivary secretions (Tatchell and Moorhouse, 1970; Berenberg et al., 1972; Theis and Budwiser, 1974) and might be a function of the salivary secretions generating an ample flow of blood for the rapid engorgement phase. However, in one case ticks have been found to feed successfully without extensive tissue destruction (Tatchell and Moorhouse, 1970) suggesting that the extensive lesions are not essential.

On primary infestation the cellular infiltrate at H. a. anatolicum feeding sites in both cattle and rabbits was dominated by neutrophils followed by mononuclear cells. However, in rabbits the infiltration of basophils and eosinophils was an early event (24 hours) whereas in cattle, they were not seen until 144 hours after attachment. The early infiltration of basophils in rabbits was intriguing. It is possible that they had been attracted by early degranulation of mast-cells. This was consistent with the findings of Brossard and Fivaz (1982) on I. ricinus feeding on rabbits. In cattle the infiltration of basophils and eosinophils appeared to follow release of mediators from degranulated mast-cells.

The histochemical nature of the cement cone of H. a. anatolicum, the presence of intact and degranulated basophils along the cement

cone on tertiary infestation, and the demonstration of an antigenic aminopeptidase component in the saliva strongly suggested that the attachment cement of H. a. anatolicum was antigenic in nature. This is consistent with the findings of Gregson (1970) who demonstrated D. andersoni cement-specific in vitro responsiveness of lymphocytes from sensitized individuals. However, this is in contrast to the observations of Tatchell and Moorhouse (1968) who claimed that the cement of B. microplus was inert in nature.

On tertiary infestation the resistance was expressed as prolonged time to engorgement, reduced intake of blood meal, failure to lay eggs and death in situ. The expression of resistance was stronger in cattle than in rabbits. Sixty-two percent of the females failed to lay eggs and 57% died in situ in cattle as compared to 14% and 7% in rabbits.

The stronger expression of resistance in cattle corresponded with pronounced epidermal vesiculation around the mouthparts of feeding H. a. anatolicum ticks and serous exudation. Riek (1956, 1962) observed similar papular reactions at B. microplus larval feeding sites on resistant cattle along with an increase in blood histamine levels. He equated the ability to produce epidermal vesicles with the degree of resistance. This was also consistent with the results obtained from skin tests with antigens isolated from the saliva of H. a. anatolicum.

All the three antigens (antigen I, molecular weight 130,000 daltons; antigen II, molecular weight 103,000 daltons; antigen III, molecular weight 96,000 daltons) isolated from saliva elicited immediate

oedematous skin reactions on intradermal inoculations into hypersensitized rabbits. This indicated an interaction between the salivary antigens and the specific homocytotropic antibodies on the surface of sensitized cells. This was supported by the presence of a large number of degranulated mast-cells and basophils (Figures 7.7 and 8.9) at H. a. anatolicum tick feeding sites. This also corroborates the development of homocytotropic antibodies (Boese, 1974; McGowan et al., 1979) and the progressive sensitization of basophils to salivary antigens (Brossard et al., 1982) during the course of multiple infestations. The allergens isolated and purified from B. microplus larvae gave similar oedematous skin reactions on intradermal inoculation into tick resistant cattle (Willadsen and Williams, 1976; Willadsen et al., 1978; Willadsen and Riding, 1979) and the level of tick resistance correlated with the immediate hypersensitivity reactions to allergens 1 and 2. In contrast, the immediate skin reactions to intradermal inoculation of D. andersoni SGA into sensitized guinea-pigs were insignificant as compared to those of naive controls (Wikel et al., 1978).

The possible mechanisms by which immediate hypersensitivity reactions effected resistance could be as follows:-

(i) Mediators released during hypersensitivity induce host grooming, which has been found to be responsible for the loss of B. microplus larvae in resistant hosts (Koudstaal et al., 1978).

(ii) Epidermal vesiculation and serous exudation results in entrapment of the ticks, causing poor feeding and death of the ticks without engorgement.

(iii) Toxic effect of mediators on the tick, interfering with salivary secretions or other physiological functions.

The comparatively weaker immediate reactions in rabbits could be due to low mast-cell density in the skin (Relay, 1959), low concentrations of salivary antigen-specific homocytotropic antibodies or the ability of the antibodies to bind to target cells (Willadsen, 1980).

Comparing the nature and magnitude of cellular responses at H. a. anatolicum feeding sites in cattle and rabbits, basophils and to a lesser extent eosinophils appeared to be the major immune effector cells of resistance. The stronger expression of resistance in cattle corresponded with a higher level of cutaneous basophilia. The reactions at H. a. anatolicum feeding sites in cattle were typical of cutaneous basophil hypersensitivity reactions. At 144 hours after attachment basophils comprised 23% of the cellular infiltrate as compared to 9% in rabbits, and were the second most abundant cell type at this stage. On the other hand the eosinophil responses in cattle were substantially weaker as compared to rabbits. In the A. americanum guinea-pig system the tick rejection coincided with the arrival and degranulation of basophils and the level of resistance was directly proportional to the magnitude of the cutaneous basophil response (Brown and Askenase, 1981). The treatment of tick resistant guinea-pigs with specific anti-basophil serum before challenge completely blocked the expression of resistance (Brown et al., 1982a) and also resulted in the reduction of the cutaneous eosinophil response at tick feeding sites. This suggested an important role of basophils in the expression of resistance against ticks.

In contrast, basophils were rarely seen at B. microplus feeding sites (Schleger et al., 1976) and there was a progressive decrease in basophil numbers at I. ricinus (Allen et al., 1977) feeding sites in cattle. On the other hand, the basophil response was of a much greater magnitude in the guinea-pig tick system (Allen, 1973; Brown and Knap, 1981; Brown et al., 1984).

The eosinophil response in rabbits was of a much greater degree than in cattle. Eosinophils were the third most abundant cell type at tick feeding sites in rabbits and formed 18-21% of the cellular infiltrate up to 72 hours after tertiary infestation. This is in line with the observations of Schleger et al. (1976) and Allen et al. (1977) on cattle and Brossard and Fivaz (1982) on rabbits. The administration of anti-eosinophil serum to tick resistant guinea-pigs before tick challenge impaired the expression of resistance (Brown et al., 1982a). How eosinophils effect resistance is not clear. The major basic protein (MBP) of eosinophil granules has been found to damage a number of parasites in vivo and in vitro (Butterworth et al., 1979; Kierszenbaum et al., 1981). However, the treatment of guinea-pigs with rabbit antibody to guinea-pig eosinophil major basic protein before and during tick challenge failed to alter the expression of resistance to A. americanum (Brown and Askenase, 1983). It is possible that basophil/mast-cell derived factors might enhance eosinophil mediated, complement-dependent damage (Anwar et al., 1980). In addition they might enhance eosinophil mediated antibody-dependent cell mediated cytotoxicity (Capron et al., 1981) to the gut epithelia of the tick. A rapid decrease in the proportion of eosinophils from

18% at 72 hours to 9% at 144 hours corresponded with their degranulation at tick feeding sites, probably to modulate the effect of histamine from degranulated mast-cells/basophils. Mast-cells and basophils are a rich source of histamine (Askenase, 1977). The degranulation of mast-cells and basophils results in increased levels of histamine and other mediators at tick feeding sites. Concurrent administration of antihistamines modified the expression of resistance (Wikel, 1982). Histamine has been found to have a direct affect on tick feeding *in vivo* (Kemp and Bourne, 1980) and *in vitro* (Paine *et al.*, 1983). The exact mechanisms by which histamine and other mediators effect resistance is not clear. However, the possible mechanisms could be as follows:-

(i) They compete with catecholamines at neuroglandular junctions as antagonists or very weak agonists and impair secretion of saliva. This is supported by the presence of increased secretory material in the salivary glands of ticks fed on resistant hosts and a significant reduction of recordings associated with sucking and salivation on addition of histamine and 5-hydroxytryptamine in the feeding medium of *D. andersoni* (Paine *et al.*, 1983) in an *in vitro* system.

(ii) The mediators released at tick feeding sites induce pruritis leading to reflex grooming by the host.

(iii) They might interact with some digestive enzymes in the saliva or in the gut and inactivate them, change their structure or affect their function.

(iv) They might have toxic affects on some vital physiological centres such as the central ganglion. The higher peristaltic rates

of midgut diverticula of D. andersoni larvae which survived and engorged on resistant hosts as compared to larvae fed on naive hosts (Allen and Kemp, 1982) was probably suggestive of a toxic effect of mediators ingested by the tick.

(v) The presence of antihistamines in the salivary glands of ticks (Chinery and Ayitey-Smith, 1977) again seems to be a mechanism to counteract the effect of host histamine.

Neutrophils remained the dominant cells in the cellular infiltrate throughout tertiary infestation in both the rabbits (42-53%) and the cattle (48-68%). The massive tissue destruction at tick feeding sites paralleled the degree of neutrophil infiltration and degranulation. It appeared likely that in addition to the inflammatory response and chemotaxis caused by salivary secretions (Berenberg et al., 1972), the formation of immune complexes might have contributed to the infiltration of neutrophils, vascular damage and tissue destruction. The production of precipitating and circulating antibodies in response to tick infestation has been reported in a number of tick-host systems (Willadsen, 1980).

The neutralization of salivary components by host antibodies appears to be another important effector mechanism of resistance. In the present study the antigenic proteins were identified by incubating salivary proteins (separated by SDS-PAGE and blotted onto nitrocellulose paper) with immune serum from naturally tick immune rabbits and then visualised by goat anti-rabbit IgG. The nine proteins identified as antigenic formed more than 95% of the salivary protein. Therefore it seemed reasonable to speculate that the

majority of the salivary enzymes would be neutralized by host antibodies at the feeding site or in the gut of the tick and might deprive the tick of essential digestive enzymes.

The other effector mechanism by which antibodies mediate resistance might be the recruitment of basophils to tick feeding sites. Resistance to A. americanum (Brown and Askenase, 1981) and R. appendiculatus (Askenase et al., 1982) was adoptively transferred with immune serum along with the ability to mount a cutaneous basophil response on challenge infestation. Fractionation of immune serum showed resistance activity to be in the IgG- and IgG1-containing fractions (Brown et al., 1982b).

Antigen II, antigen III, whole saliva and SGE from H. a. anatolicum also elicited strong delayed skin reactions in hypersensitized rabbits on intradermal inoculation. The reactions reached their peak levels at 24 hours after inoculation and then subsided gradually. Wikel et al. (1978) and Wikel and Osburn (1982) reported similar skin reactions to D. andersoni SGA in tick resistant guinea-pigs and Bos taurus calves. The lymphocytes from these hosts also displayed SGA-specific in vitro lymphocyte blastogenesis. However, allergens isolated from B. microplus larvae failed to elicit any delayed skin reactivity (Willadsen and Williams, 1976; Willadsen et al., 1978; Willadsen and Riding, 1979). Collins et al. (1970) reported delayed skin reactions due to the presence of homocytotropic antibodies at skin test sites, and such a possibility cannot be ruled out. However, resistance to D. andersoni (Wikel, 1976) and A. americanum (Brown, 1982) was adoptively transferred between inbred guinea-pigs with

sensitized lymph node cells (more readily with T-cell enriched populations) and peritoneal exudate cells respectively, as were the cutaneous basophil reactions. This further suggested the involvement of cellular components in the recruitment of basophils to tick feeding sites in resistant hosts. The results of the present study suggest that the immune serum components act in concert with immune cells to produce cellular reactions inimical to ticks.

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APPENDIX 1

1.1 Glutaraldehyde (25%) 10 ml
Formaldehyde 36% 5.55 ml
0.2M Phosphate buffer, pH 7.2 50 ml
Made up to 100 ml with distilled water

1.2 2-Hydroxyethyl methacrylate 300 ml
Amberlyst A-21 100 g
Charcoal 15 g
Anhydrous sodium sulphate 50 g

Mixed and shaken for 48 hours at room temperature, filtered through Whatman's filter paper (No. 2) and its pH adjusted to 7.0-7.4. It was stored at 4°C in air tight bottles in the dark until required.

1.3 Benzoyl peroxide 0.35 g
Polyethylene glycol 4.0 ml
To mix, stir for two hours at 50°C.

1.4 Basic fuchsin 1 g
Distilled water 200 ml
N-Hydrochloric acid 20 ml
Sodium metabisulphate 1 g
Activated charcoal 2 g

Basic fuchsin was dissolved in distilled water by boiling (with occasional shaking) for 5 minutes and then allowed to cool. When the temperature was down to 50°C, the solution was filtered and N-HCl added, cooled to 25°C and sodium metabisulphate added to it. Stored in dark overnight. To this solution was added 2 g activated charcoal, shaken for one minute, filtered and stored in a dark bottle at 4°C.

1.5 Non specific esterases

1.5.1 α-Naphthyl acetate 5 mg
Acetone 0.1 ml
0.2M phosphate buffer, pH7.4 10 ml
Fast Blue B 30 mg

The α-Naphthyl acetate was dissolved in the acetone and the phosphate buffer added and thoroughly mixed. The Fast Blue B was added and the solution filtered and used immediately.

1.5.2 5-Bromo-4-chloro-indoxyl acetate 1 mg
Ethanol 0.1 ml
0.2M Tris buffer, pH 7.2 2 ml
Potassium ferricyanide (0.05M)(1.6%) 17 mg
Potassium ferrocyanide (0.05M)(2.1%) 21 mg
Calcium chloride (0.1M) (2.1%) 11 mg
Distilled water 7.9 ml

The 5-bromo-4-chloro-indoxyl acetate was dissolved in the ethanol and the buffer was then added. The remaining chemicals were dissolved in distilled water and the solution was mixed. It is important that the solution is freshly prepared.

1.6 Incubating medium:

Substrate solution*	0.5 ml
0.1M Acetate buffer, pH 6.5	5 ml
0.85% sodium chloride	4 ml
Potassium cyanide (65mg/50ml)	0.5 ml
Fast Blue B salt	5 mg

*Substrate solution:

L-leucyl-4-methoxy Beta-naphthylamide	4 mg
Ethyl alcohol	0.1 ml
Distilled water	4.9 ml

1.7 Naphthol AS-B1-β-D-glucosiduronic acid 11 mg
0.05M Sodium bicarbonate 1.0 ml

The solution was made to 100 ml with 0.1M acetate buffer (pH 4.5). For use, further dilute (1:1) with 0.1M acetate buffer, pH 4.5.

1.8 Incubating medium:

Substrate solution*	10 ml
Sodium chloride	260 mg
Hexazonium pararosanilin**	0.6 ml

*Naphthol AS sulphate (Potassium salt) 20 mg
0.85% Sodium chloride 8 ml
0.2M Acetate buffer (1.64 g sodium acetate, anhydrous in 100 ml) 2 ml

**Pararosanilin hydrochloride 0.3 ml
Fresh 4% sodium nitrite 0.3 ml

Preparation of hexazotized pararosanilin:
Solution 1.

Pararosanilin hydrochloride	1 g
Distilled water	20 ml
HCl (Conc)	5 ml

The paraosanilin is dissolved in distilled water and the HCl was added. The solution was heated gently, cooled, filtered and stored at 4°C.

Solution 2.
1% sodium nitrite

Solution 1. and 2. in equal parts, allowed to stand for 30 seconds until the solution becomes amber.

1.9 Phosphatases

1.9.1

Sodium α-naphthyl phosphate	10 mg
0.1M Tris buffer, pH 10.0	10 ml
Fast Red TR	10 mg

The sodium α-naphthyl phosphate was dissolved in the buffer. Fast Red TR was added and the solution well mixed. The final pH of the incubating medium was between 9.0 and 9.4.

1.9.2

2% Sodium beta-glycerophosphate	2.5 ml
2% Sodium veronal	2.5 ml
2% Calcium nitrate	5.0 ml
1% Magnesium chloride	0.25 ml
Distilled water	1.25 ml

The final pH of the incubating medium was between 9.0 and 9.4.

1.9.3

Sodium α-naphthyl phosphate	10 mg
0.1M Acetate buffer, pH 5.0	10 ml
Fast Garnet GBC	10 mg

The sodium α-naphthyl phosphate was dissolved in the buffer, the diazonium salt was then added. The solution was then filtered and used immediately.

1.9.4

Sodium β-glycerophosphate	32 mg
0.5M Veronal acetate buffer, pH 5.0	10 ml
Lead nitrate	20 mg

The lead nitrate was dissolved in buffer before β-glycerophosphate was added to it. The pH of the incubating medium was approximately 5.0.

1.9.5

0.125% Adenosine triphosphate	4 ml
0.2M Tris buffer, pH 7.2	4 ml
2% Lead nitrate	0.6 ml
2.5% Magnesium nitrate	1 ml
Distilled water	0.4 ml

1.9.6

0.125% Glucose-6-phosphate	4 ml
0.2M Tris maleate, pH 6.7	4 ml
2% Lead nitrate	0.6 ml
Distilled water	1.4 ml

1.10 Oxidoreductases

1.10.1

1. Hydroxy-2-naphthoic acid	10 mg
N-Phenyl-p-phenylenediamine	10 mg
Absolute alcohol	0.5 ml
Distilled water	35 ml
0.2M Tris buffer, pH 7.4	15 ml

The first two reagents were dissolved in the absolute alcohol and then the distilled water and the buffer were added.

1.10.2 D.L. 3:4-Dihydroxyphenylalanine 100 mg
0.1M Phosphate buffer, pH 7.4 100 ml

1.10.3 Tryptamine hydrochloride 25 mg
Sodium sulphate 4 mg
Tetranitro-blue tetrazolium 5 mg
0.1M Phosphate buffer, pH 7.6 5 ml
Distilled water 15 ml

1.10.4 Incubating medium:
Succinate substrate solution* 0.9 ml
Stock tetrazolium solution** 0.1 ml

*Sodium succinate 6.75 g
Distilled water 8.0 ml
N-HCl 0.05 ml

The sodium succinate was dissolved in distilled water, the N-HCl was added and the solution tested for pH (adjusted to 7.1 if necessary) and the total volume made up to 10 ml. Stored at -20°C.

**NBT (nitro-blue tetrazolium), 4 mg/ml 2.5 ml
0.2M Tris, pH 7.4 2.5 ml
0.05M Magnesium chloride 1.0 ml
Distilled water 3.0 ml
pH was adjusted to 7.0 if necessary using stock buffer.

1.10.5 Stock tetrazolium (MTT) solution* 0.9 ml
Distilled water 0.1 ml
Co-enzyme: NADH 2.0 mg

The co-enzyme was added to 1 ml of the medium. The pH was adjusted to 7.1 with N-HCl if necessary.

*MTT (1mg/ml distilled water) 2.5 ml
0.2M Tris buffer, pH 7.4 2.5 ml
0.5M Cobalt chloride 0.5 ml
0.5M Magnesium chloride 1.0 ml
Distilled water 2.5 ml
The pH was checked and adjusted to 7.0, if necessary.

1.10.6 Stock tetrazolium solution (Appendix 1.10.4) 0.9 ml
Distilled water 0.1 ml
NADPH 2.0 mg

The co-enzyme was added just before use. The pH was checked and adjusted to 7.1, if necessary.

1.11 Glycoprotein staining

1.11.1 Di-sodium tetraborate 9.5 g
Distilled water 480 ml
Glutaraldehyde (EM grade 25%) 20 ml

1.11.2 A) Silver nitrate 1 g
Distilled water 30 ml

B) 30% Ammonium hydroxide 3.7 ml
0.8N Sodium hydroxide 5.0 ml
Distilled water 200 ml

Solutions A and B (1:1, v/v) were mixed thoroughly by stirring for 15 minutes.

1.11.3 Formaldehyde (38%) 250 µl
Citric acid 60 mg
Water 800 ml
Ethanol 100 ml

The formaldehyde and citric acid were dissolved in water and ethanol added to the solution. The solution was then made to one litre with water.

APPENDIX 2

2.1 Mast-cells, basophils, eosinophils, neutrophils and mononuclear cells in the dermis of naive rabbits (controls) and at *H. a. anatolicum* feeding sites.

2.1.1 Naive rabbits (controls)

Rabbit No.	Biopsy No.	Number of cells/20 fields				
		Mast-cells	Baso-phils	Eosino-phils	Neutro-phils	Mononuclear cells
R-15	883	16	-	1	1	27
	887	17	-	-	1	12
	890	5	-	1	-	16
R-16	897	13	-	-	-	24
	899	18	-	1	1	6
R-17	978	9	-	1	1	17
	979	11	-	2	1	13
	980	15	-	-	-	15

2.1.2 Primary infestation

2.1.2.1 Female feeding sites

Rabbit No.	Time after attachment (hours)	Biopsy No.	Number of cells/20 fields				
			Mast-cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
R-4	24	962	3	0	31	225	40
		963	3	0	39	297	80
		964	2	2	12	75	39
		965	5	1	19	219	58
R-5	24	1124	7	0	34	267	66
		1125	8	14	79	385	90
		1127	8	6	14	252	89
		1128	13	14	39	88	62
		1131	18	35	12	89	47
		1132	14	20	19	158	61
R-6	24	1446	7	23	133	754	240
		1447	10	25	164	747	218
		1449	13	20	131	738	437
		1451	7	23	66	504	256
R-7	72	971	5	0	43	682	258
		972	4	0	82	807	239
		973	0	2	28	364	175
		982	11	4	85	530	205
		983	4	2	41	497	116
R-8	72	1141	10	21	88	557	175
		1144	6	18	16	542	173
		1145	5	19	109	411	217
		1148	7	15	22	730	199
		1149	11	14	77	614	146
R-9	72	1467	6	22	93	434	208
		1474	9	26	176	626	376
		1470	13	58	356	1199	233
		1172	4	49	136	783	302
R-10	144	987	7	12	71	1122	362
		988	6	5	131	924	227
		989	9	12	79	1269	291
		990	0	6	19	143	39
R-11	144	1158	7	5	29	179	71
		1160	10	12	130	1319	399
		1164	10	21	84	717	357
		1166	13	8	72	866	347
R-12	144	1189	1	17	12	573	197
		1193	6	43	106	1145	519
		1197	12	35	59	971	449
		1201	5	26	45	842	438
		1203	4	65	112	1073	503

2.1.2.2 Male feeding sites

Rabbit No.	Time after attachment (hours)	Biopsy No.	Number of cells/20 fields				
			Mast cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
R-4	24	966	1	2	15	117	32
		967	2	0	16	15	7
		968	8	0	18	404	108
		969	8	6	15	73	24
R-5	24	1111	16	8	81	160	50
		1114	22	2	18	134	47
		1118	16	11	13	9	24
		1119	19	13	21	181	97
		1120	8	3	15	48	18
		1121	6	5	8	25	12
R-6	24	1454	15	23	112	480	142
		1457	21	36	71	548	186
		1458	10	21	175	610	156
		1460	13	47	141	685	227
		1462	16	35	67	681	263
		1463	13	19	60	587	175
		1464	11	10	188	722	288
R-7	72	974	8	2	16	197	62
		975	7	2	11	77	36
		976	3	6	14	110	22
		977	2	8	16	138	80
		985	10	1	28	195	117
		986	4	2	62	126	85
R-8	72	1151	7	7	59	102	72
		1152	6	20	92	469	258
		1155	3	16	27	386	197
		1156	5	13	58	408	129
R-9	72	1481	9	51	279	695	345
		1483	5	36	188	483	217
		1485	15	18	170	626	317
		1486	7	37	234	491	287
		1487	9	29	101	414	318
R-10	144	991	3	3	20	980	187
		992	6	3	139	389	177
		993	11	4	64	250	73
		994	6	2	44	801	217
R-11	144	1175	2	23	68	396	249
		1180	7	3	117	699	346
		1181	7	10	92	755	350
		1182	5	32	143	1596	290
		1183	4	17	105	1067	360
		1185	6	16	89	744	365
R-12	144	1205	9	62	138	1123	509
		1207	11	38	133	1120	414
		1208	8	47	71	1034	545
		1211	13	28	31	603	295
		1218	6	57	100	874	395

2.1.3 Tertiary infestation

2.1.3.1 Female feeding sites

Rabbit No.	Time after attachment (hours)	Biopsy No.	Number of cells/20 fields				
			Mast-cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
R-21	24	1615	2	85	320	594	364
		1616	1	48	256	1069	257
		1617	0	45	395	1123	422
		1619	4	84	345	1416	299
		1623	5	72	318	1395	652
		1624	5	34	277	695	239
R-22	24	1661	0	28	302	1167	321
		1662	4	102	718	616	303
		1658	3	42	184	1106	420
		1665	6	46	174	630	408
		1666	2	101	564	1062	360
R-23	24	1707	2	152	713	899	581
			1	68	355	907	480
			6	152	443	1197	503
			2	101	291	818	407
			1	136	539	1047	691
R-24	72	1678	2	67	121	563	483
		1685	1	90	96	475	448
		1686	0	52	141	529	485
		1687	2	156	661	790	490
		1695	0	214	199	497	449
R-25	72	1733	4	64	155	533	488
		1735	1	74	68	490	402
		1737	3	97	135	599	432
		1741	0	160	120	368	283
		1745	2	193	349	888	436
		1746	1	155	232	291	270
R-26	72	1635	2	113	187	425	393
		1636	0	92	263	885	412
		1638	2	32	478	634	468
		1639	1	82	309	475	302
		1641	4	75	133	247	308
		1642	0	20	502	471	352
R-28	144	1851	2	105	138	423	504
		1852	0	44	193	603	504
		1856	1	208	79	602	433
		1860	1	101	52	756	477
		1861	2	125	414	819	548
		1862	0	58	165	411	479
R-29	144	1874	2	79	66	569	370
		1879	1	161	166	861	454
		1881	3	18	79	771	435
		1882	1	84	106	799	772
		1876	1	119	94	541	612
R-27	144	1832	0	174	96	433	407
		1833	1	131	83	249	364
		1835	1	147	61	306	468
		1838	0	93	54	379	283
		1841	0	65	60	206	226

2.1.3.2 Male feeding sites

Rabbit No.	Time after attachment (hours)	Biopsy No.	Number of cells/20 fields				
			Mast-cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
R-21	24	1625	4	16	92	436	230
		1628	2	87	348	1255	356
		1629	2	61	376	675	465
		1632	6	71	385	976	618
		1633	1	29	94	423	385
R-22	24	1669	3	31	450	1021	310
		1671	0	82	313	821	330
		1672	1	32	338	508	347
		1677	1	34	190	1154	476
		1661	5	49	218	970	622
R-23	24	1720	3	116	89	1249	539
		1723	3	30	134	1400	630
		1724	9	87	262	645	342
		1725	3	99	210	1385	860
		1732	5	81	129	865	547
R-25	72	1750	0	128	274	407	364
		1751	2	127	391	364	372
		1752	5	67	126	537	445
		1756	2	56	98	201	269
		1759	4	73	120	486	372
R-26	72	1646	2	86	416	571	499
		1648	2	172	204	535	490
		1649	0	54	211	824	391
		1651	0	42	699	1153	378
		1656	3	42	164	1207	320
R-24	72	1697	1	55	261	1649	529
		1698	2	111	361	1030	568
		1699	4	121	687	1799	500
		1702	3	86	139	271	239
		1705	2	92	76	612	405
R-27	144	1843	0	105	60	405	540
		1845	1	179	59	616	462
		1846	2	99	141	747	463
		1847	1	105	49	163	456
		1848	0	113	78	828	712
		1849	3	70	88	234	200
R-28	144	1863	2	84	86	725	317
		1864	2	127	77	369	347
		1867	1	117	160	651	326
		1868	1	121	30	1035	475
		1872	0	76	290	996	399
R-29	144	1884	2	116	284	1156	707
		1885	1	104	163	871	472
		1888	2	64	70	799	435
		1889	0	79	104	575	312
		1890	1	123	119	1112	468

2.2 Time to engorgement, engorged weight and oviposition in female H. a. anatolicum fed on rabbits.

2.2.1 Primary infestation

Rabbit No.	Female	Time to engorgement (days)	Weight of fed female (mg)	Weight of egg mass (mg)
R-107	A	7	365	204
	B	8	488	270
	C	8	224	117
	D	8	325	134
	E	8	286	127
	F	8	487	313
	G	8	298	159
	H	9	355	199
	I	9	306	167
	J	9	344	187
R-108	A	7	489	265
	B	7	219	110
	C	7	491	218
	D	8	525	312
	E	8	408	203
	F	8	334	166
	G	8	310	126
	H	8	618	379
	I	8	328	162
	J	9	235	127
R-109	A	7	326	182
	B	7	440	253
	C	7	461	257
	D	7	469	309
	E	7	196	78
	F	7	211	115
	G	8	278	164
	H	8	259	107
	I	9	608	378
	J	9	324	158
R-110	A	7	459	277
	B	7	407	234
	C	7	475	280
	D	7	309	164
	E	7	289	144
	F	7	430	207
	G	8	363	199
	H	8	295	-
	I	9	384	210
	J	9	282	109

2.2.1 (continued)

Rabbit No.	Female	Time to engorgement (days)	Weight of fed female (mg)	Weight of egg mass (mg)
R-111	A	7	271	738
	B	8	353	191
	C	8	299	168
	D	8	369	223
	E	8	211	103
	F	8	240	114
	G	8	208	97
	H	9	259	182
	I	9	332	169
	J	9	290	142
R-112	A	7	455	222
	B	8	489	315
	C	8	473	269
	D	7	309	156
	E	8	216	107
	F	8	324	188
	G	8	297	166
	H	8	412	227
	I	7	260	146
	J	7	415	227

2.2.2 Tertiary infestation

Rabbit No.	Female	Time to engorgement (days)	Weight of fed female (mg)	Weight of egg mass (mg)
107	A	9	197	59
	B	9	114	36
	C	10	151	47
	D	11	65	14
	E	11	69	19
	F	12	228	92
	G	12	84	15
	H	12	160	48
	I	13	104	37
	J	14	92	24
108	A	8	165	62
	B	8	135	51
	C	9	112	22
	D	9	84	34
	E	10	79	27
	F	11	47	14
	G	11	65	-
	H	12	102	48
	I	12	62	21
	J	12	72	-
109	A	9	152	71
	B	9	133	49
	C	9	165	64
	D	9	71	24
	E	10	146	64
	F	10	125	35
	G	13	67	-
	H	14	31	-

- Died in situ

APPENDIX 3

3.1 Mast-cells, basophils, eosinophils, neutrophils and mononuclear cells in the dermis of cattle in control biopsies and at H. a. anatolicum feeding sites.

3.1.1 Controls

3.1.1.1 Primary controls

Calf No.	Biopsy No.	Number of cells/20 fields				
		Mast cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
45	2441	16	-	-	-	20
	2442	15	-	-	-	32
46	2453	15	-	-	-	29
	2454	17	-	-	-	27
107	2057	28	-	-	-	41
	2058	46	-	-	-	66
108	2059	19	-	-	-	26
	2060	13	-	-	-	28

3.1.1.2 Tertiary controls

45	2508	33	-	-	-	20
	2509	54	-	-	-	30
46	2510	42	-	11	-	21
	2511	47	-	2	-	38
107	2258	63	-	20	1	31
	2259	42	-	-	-	54
108	2260	26	-	-	-	44
	2261	20	1	5	-	32

3.1.2 Primary infestation

Calf No.	Time after attachment (hours)	Biopsy No.	Number of cells/20 fields				
			Mast cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
45	24	2443	12	-	-	21	22
		2444	9	-	-	15	11
		2445	1	-	-	9	9
46	24	2454	9	-	-	21	39
		2455	11	-	-	94	47
		2456	11	-	-	12	12
107	24	2061	20	-	-	17	15
		2062	20	-	-	4	8
		2063	21	-	-	7	33
108	24	2064	7	-	-	14	23
		2065	11	-	-	340	25
		2066	7	-	-	371	63

3.1.2 (continued)

Calf No.	Time after attachment (hours)	Biopsy No.	Number of cells/20 fields				
			Mast-cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
45	72	2446	6	-	-	358	168
		2447	6	-	-	334	183
		2448	14	-	-	330	167
46	72	2457	5	-	2	299	116
		2458	8	-	-	429	149
		2459	6	-	-	256	114
107	72	2067	9	2	-	432	90
		2068	22	11	-	205	63
		2069	22	5	-	243	47
108	72	2070	7	1	-	320	127
		2071	8	-	-	646	211
		2072	2	1	-	59	65
45	144	2449	5	9	1	357	177
		2450	11	19	2	584	240
		2451	5	-	-	257	111
46	144	2460	2	10	6	438	311
		2461	9	37	1	1036	199
		2462	12	19	2	581	217
107	144	2073	3	24	1	379	157
		2074	1	19	2	421	194
		2075	4	44	1	624	229
108	144	2076	16	16	-	272	68
		2077	8	8	-	465	115
		2078	39	39	6	758	144

3.1.3 Tertiary infestation

45	24	2512	-	6	24	699	166
		2513	1	14	15	457	127
		2514	3	19	30	632	160
46	24	2515	-	135	48	1360	254
		2516	4	161	15	644	136
		2517	1	333	94	394	113
107	24	2262	10	1	1	195	91
		2263	11	7	3	266	244
		2264	11	40	22	236	145
108	24	2265	5	7	11	250	170
		2266	3	3	17	266	119
		2267	4	4	6	745	142
45	72	2518	4	72	42	1265	184
		2519	19	31	14	1233	127
		2520	-	140	70	1510	109
46	72	2521	-	641	71	532	189
		2522	-	565	100	1342	225
		2523	-	422	34	1562	177

3.1.3 (continued)

Calf No.	Time after attachment (hours)	Biopsy No.	Number of cells/20 fields				
			Mast-cells	Basophils	Eosinophils	Neutrophils	Mononuclear cells
107	24	2268	1	353	29	1297	330
		2269	2	686	146	1150	385
		2270	1	377	70	1135	442
108	24	2271	2	105	39	1504	284
		2272	2	89	24	1242	289
		2273	10	235	35	1123	271
45	144	2524	-	277	118	765	193
		2525	-	114	86	835	286
		2526	-	335	34	973	117
46	144	2527	-	785	405	710	374
		2528	-	521	181	972	298
		2529	1	814	272	631	321
107	144	2277	6	422	35	729	332
		2278	5	707	58	909	408
		2282	4	768	375	730	482
108	144	2283	-	83	39	530	246
		2287	4	256	58	630	305
		2291	2	77	143	292	338

3.2 Time to engorgement, engorged weight and oviposition in female H. a. anatolicum fed on cattle.

3.2.1 Primary infestation

Calf No.	Female	Time to engorgement (days)	Weight of fed female (mg)	Weight of egg mass (mg)
45	A	6	554	320
	B	6	561	341
	C	6	476	312
	D	6	399	225
	E	6	396	265
	F	7	478	284
	G	7	632	299
	H	7	541	287
46	A	6	339	205
	B	6	496	332
	C	6	511	-
	D	6	431	238
	E	6	521	340
	F	6	527	325
	G	6	472	307
	H	6	504	299
	I	6	336	196
	J	6	330	205
107	A	5	529	357
	B	6	370	239
	C	6	380	235
	D	6	581	341
	E	7	480	283
	F	7	387	143
	G	7	421	243
	H	7	492	310
108	A	7	447	320
	B	7	488	-
	C	7	304	172
	D	7	395	234
	E	8	554	314
	F	8	443	194
	G	8	345	175
	H	8	442	232

3.2.2 Tertiary infestation

Calf No.	Female	Time to engorgement (days)	Weight of fed female (mg)	Weight of egg mass (mg)
45	A	6	45	-
	B	6	23	-
	C	7	16	-
	D	7	234	122
	E	8	195	98
	F	8	111	-
	G	9	90	18

H to L died on the host without engorgement.

46	A	8	55	-
	B	8	99	5*
	C	8	108	30*
	D	8	115	-
	E	8	38	-
	F	8	36	-

G to L died on the host.

*Eggs did not hatch.

APPENDIX 4

Skin reactivity (mean diameter [mm] ± S.D.) of hypersensitized rabbits to intradermal inoculation of salivary antigens

Antigen	15 minutes	30 minutes	60 minutes	2 hours	4 hours	24 hours	48 hours
Antigen I	7.38 ± 0.5 (n = 4)	8.5 ± 0.58 (n = 4)	11.5 ± 0.58 (n = 4)	10.25 ± 0.5 (n = 4)	9.0 ± 1.0 (n = 3)	6.3 ± 2.3 (n = 3)	4.3 ± 0.58 (n = 3)
Antigen II	8.25 ± 1.5 (n = 4)	9.25 ± 0.96 (n = 4)	11.25 ± 0.96 (n = 4)	12.25 ± 1.26 (n = 4)	10.33 ± 0.58 (n = 3)	12.5 ± 0.5 (n = 3)	11.67 ± 1.53 (n = 3)
Antigen III	9.75 ± 0.5 (n = 4)	11.9 ± 0.6 (n = 4)	13.25 ± 0.5 (n = 4)	13.5 ± 1.29 (n = 4)	13.3 ± 1.5	20.67 ± 4.16 (n = 3)	16.67 ± 4.16 (n = 3)
Saliva	10.75 ± 0.96 (n = 4)	14.0 ± 1.4 (n = 4)	16.0 ± 1.6 (n = 4)	18.75 ± 1.89 (n = 4)	19.3 ± 0.58 (n = 3)	31.3 ± 8.08 (n = 3)	28.5 ± 11.03 (n = 3)
SGE	11.0 ± 2.16 (n = 4)	13.25 ± 1.19 (n = 4)	17.25 ± 1.5 (n = 4)	20.5 ± 2.08 (n = 4)	19.5 ± 0.5 (n = 3)	36.0 ± 10.15 (n = 3)	30.3 ± 13.05 (n = 3)
PBS + acrylamide	6	6.25 ± 0.5 (n = 4)	5.5 ± 0.58 (n = 4)	5.25 ± 0.5 (n = 4)	5.0 (n = 3)	4.0 (n = 3)	3.17 ± 0.76 (n = 3)
PBS	5.5 ± 1.0 (n = 4)	5.0 ± 0.82 (n = 4)	4.25 ± 1.26 (n = 4)	3.5 ± 0.58 (n = 4)	3.0 (n = 3)	-	-

SGE = Salivary gland extracts; PBS = Phosphate buffered saline; n = number of replicates.

APPENDIX 5

Suppliers of Chemicals and Equipment Used in this Study

Agar Aids,
662a Cambridge Road,
STANSTEAD, Essex, England.

Bayer U.K. Ltd.,
Agrochem Division,
Eastern Way,
BURY ST. EDMUNDS, Suffolk, England.

Cambridge Instruments Ltd.,
Rustat Road,
CAMBRIDGE, England.

EM Scope Labs. Ltd.,
Kingsworth Industrial Estate,
Walton Road,
ASHFORD, Kent, England.

Fisons,
LOUGHBOROUGH, England.

Johnson Matthey Chemicals Ltd.,
74 Hatton Gardens,
LONDON, England.

Koch-Light Laboratories Ltd.,
Poyle Estate,
Willow Road,
Colnbrook,
SLOUGH, Berkshire, England.

E. Leitz Wetzler,
GmbH, Postfach 2020,
D-6330 WETZLER, West Germany.

Miles Laboratories Ltd.,
P.O. Box 37,
Stoke Court, Stoke Poges,
SLOUGH, Berkshire, England.

National Diagnostics,
198 Route 206 South,
SOMERVILLE, New Jersey, U.S.A.

Polaron Equipment Ltd.,
21 Greenhill Crescent,
Holywell Industrial Estate,
WATFORD, Hertfordshire, England.

Raymond A. Lamb,
6 Sunbeam Road,
North Acton, LONDON.

Sigma Chemical Co. Ltd.,
Fancy Road,
POOLE,
Dorset, England.

Taab Laboratories Ltd.,
40 Grovelands Road,
READING, Berkshire,
England.

Addendum

Tables 5.3 and 5.4

The intensity of reaction represents the approximate intensity within any specified cell/granule type and the sequence indicates a decrease or increase in the intensity of reaction during feeding.

In both the male and female ticks * indicates the reaction intensity observed in the salivary glands of unfed ticks while ** indicates the reaction intensity at 144 hours except for the secretory granules of a, d, e and f cells in females. In female a, d and e cell granules, ** indicates the intensity of the reaction observed at 72 hours and in f cell * indicates the reaction at 24 hours and ** at 72 hours post attachment.

Table 5.5

* indicates the reaction intensity observed at 24 hours while ** indicates the reaction intensity at 144 hours post attachment. The sequence indicates a decrease or increase in the intensity of reaction during feeding.

Tables 7.1, 7.3 and 8.1

Massive tissue necrosis towards the end of primary infestation and throughout tertiary infestation made it impossible to score areas immediately next to the cement cone and below the mouthparts. No attempt was therefore made to score such fields. In addition, the cells which were difficult to identify due to necrosis or degranulation in the fields scored were not included on the counts. Therefore, the quantification of cell types represents identifiable cells only.

Acarology VI (1984), 1: 365

7.5 PRELIMINARY HISTOCHEMICAL STUDIES ON THE SALIVARY GLANDS OF UNFED AND FEEDING *HYALOMMA ANATOLICUM ANATOLICUM*

H. S. Gill and A. R. Walker†

For a better understanding of the development and release of tick-transmitted pathogens, it is essential to know what changes take place in the salivary glands of feeding ticks and the reactions at tick-bite lesions. To this end a preliminary investigation of the sequential histochemical changes occurring in the salivary glands of feeding *H.a. anatolicum* was undertaken.

Salivary glands of both male and female ticks, uninfected or infected with *Theileria annulata* were embedded in methacrylate, and semi-thin sections stained with Giemsa for conventional histology. Specific histochemical methods for polysaccharides, proteins, lipids, and enzymes were employed to determine the nature of the potential secretory material in the salivary glands. Cryostat sections and whole salivary glands were used to confirm some histochemical observations.

Type I acini are non-granular. Their cells lack individual cell boundaries. These acini remain constant morphologically and histochemically throughout feeding. Type II acini contain at least 6 granular cell types – *a*, *b*, and c_1 –c_4. In feeding ticks there is a slight decrease in the granules of *a* cells. The granules of *b* and *c* cells remain almost constant. Interstitial cells increase slightly. Type III acini contain 3 granular cell types – *d*, *e*, and *f*. During feeding there is a slight decrease in the *d* cell granules, whereas *e* cell granules almost vanish towards the end of feeding. Activity in the interstitial and *f* cells is increased enormously with feeding (a water secretory function has been described by other workers). Type IV acini, present only in males, have only one granular cell type '*g*'. There is an enormous increase in these granules towards the end of feeding.

Most granule types give multiple histochemical reactions indicating their complex composition. The relationship between the granules found and the production of saliva is not obvious. Histochemistry of tick-bite lesions and the biochemical analysis of saliva is required in order to compare with the results obtained from examination of the salivary glands. Further study by electron microscopy is required to indicate the mode of secretion of these granules.

It is *b* or *c* cells of type II acini, and *e* cells of type III acini, which are found infected with *T. annulata*.

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PROCEEDINGS OF THE BRITISH SOCIETY FOR PARASITOLOGY

Spring Meeting held at the University of Bristol, 4–6 April 1984

Histological Analysis of Tick Feeding Sites During Acquisition of Resistance to *Hyalomma anatolicum anatolicum*. By H. S. Gill and A. R. Walker (*Centre for Tropical Veterinary Medicine, University of Edinburgh*)

The histological picture of the tick feeding sites following primary infestation agrees with the findings of Tatchell & Moorhouse (*Science* (1970), **167**, 1002–3) that vascular damage is caused by the tick whilst tissue damage is caused by the host response. Quantitation of cellular response indicated that the neutrophils were the predominant cells throughout feeding, followed by mononuclear cells and eosinophils. Basophils were observed in most of the tick feeding sites but always in small numbers. The lesions on resistant hosts were characterized by massive degranulation of mast cells and basophils. Localized eosinophil aggregation especially at the periphery of the feeding lesion was a constant feature of 24 h feeding lesions. As the feeding advanced there was an increase in the proportion of infiltrating mononuclear cells and basophils and a decrease in the proportion of eosinophils. Neutrophils remained the predominant cells throughout tertiary feeding. There was no significant difference in the proportion of infiltrating cells in the male and female tick feeding sites, although the lesions produced by female feedings were of far larger size than those of males. The nature and sequence of cellular events in sensitized hosts suggests the involvement of multiple hypersensitivity reactions in the expression of resistance. Pharmacological mediators released by degranulating basophils and eosinophils and necrosis of the inflammatory focus could be the main cause of reduced feeding by ticks.